Departamento de Fisiología

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Modificaciones de la función de la inervación perivascular en arteria mesentérica superior en distintas situaciones fisiológicas y patológicas

TESIS DOCTORAL

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CERTIFICAN, que Doña Esther Sastre Gil ha realizado bajo su dirección el presente trabajo ***"Modificaciones de la función de la inervación perivascular en arteria mesentérica superior en distintas situaciones fisiológicas y patológicas"*** como Tesis para alcanzar el grado de Doctor por la Universidad Autónoma de Madrid.

Para que conste a efectos oportunos, expiden y firman la presente en Madrid a 14 de Septiembre de 2015.

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Abreviaturas

ACh. Acetilcolina

ATP: Adenosín trifosfato

BH_4: Tetrahidrobiopterina

CaM: Calmodulina

cAMP: Adenosín monofosfato cíclico

cGMP: Guanosín monofosfato cíclico

CGRP: Péptido relacionado con el gen de la calcitonina

CL: Calcitonin-like receptor

COMT: Catecol-metil transferasa

COX: Ciclooxigenasa

DAG: Diacilglicerol

DEA-NO: Dietilamina NONOato

EE: Estimulación eléctrica

eNOS: Óxido nítrico sintasa endotelial

FAD: dinucleótido de flavina y adenina

FMN: mononucleótido de flavina

iNOS: Óxido nítrico sintasa inducible

IP_3: Inositol – 1,4,5- trifosfato

K_{ATP}: Canales de potasio dependientes de ATP

L-NAME: N^{ω}-nitro-L-arginina metil éster

LPS: Lipopolisacárido

MAO: Monoamino oxigenasa

NA: Noradrenalina

NADPH: nicotinamida adenina dinucleótido fosfato

NANC: Nervios no colinérgicos no adrenérgicos

NKA: Neuroquinina A

nNOS: Óxido nítrico sintasa neuronal

NO: Óxido nítrico

NPY: Neuropéptido Y

$O_2^{\cdot -}$: Anión superóxido

$ONOO^-$: Anión peroxinitrito

PGE_2: Prostaglandina E_2

PGI_2: Prostaciclina

PIP_2: Fosfatidil inositol -4, 5-bifosfato

PKA: Proteína quinasa A

PKC: Proteína quinasa C

PKG: Proteína quinasa G

PLC: Fosfolipasa C

RAMP1: receptor activity modifying protein 1

RCP: Receptor component protein

ROS: Especies reactivas de oxígeno

SHR: Ratas espontáneamente hipertensas

SNP: Nitroprusiato sódico

SP: Sustancia P

TXA_2: Tromboxano A_2

Resumen

Resultados previos han puesto de manifiesto alteraciones en la función de la inervación perivascular en arteria mesentérica superior en distintas situaciones fisiológicas y patológicas, como en hipertensión, cirrosis y envejecimiento.

El objetivo de este trabajo ha sido analizar los posibles cambios en la participación de la inervación perivascular simpática, nitrérgica y sensitiva en esta arteria en las situaciones fisiológicas: gestación y lactancia; y patológicas: hipertensión portal, hipertensión en descendencia adulta de madres diabéticas y efectos del lipopolisácarido (LPS). Asimismo se analizado si el ejercicio físico moderado revierte o previene las alteraciones en la función de la inervación perivascular en ratas espontáneamente hipertensas (SHR) y ratas sometidas a una dieta rica grasa, respectivamente. Adicionalmente se ha valorado el efecto de distintos estabilizadores de células cebadas, ketotifen y tranilast, sobre la función neuronal en dicha arteria; así como del tranilast sobre la función endotelial en arterias mesentéricas de resistencia.

Los resultados obtenidos en arteria mesentérica superior de rata son:

1. En ratas gestantes, día 20 de gestación, se produce una disminución en la respuesta vasoconstrictora inducida por estimulación eléctrica (EE) que es resultado neto de una disminución tanto en la liberación como en la respuesta a noradrenalina (NA); y un incremento en la liberación de óxido nítrico (NO) neuronal mientras que la respuesta vasodilatadora al donante de NO, dietilamina NONOato (DEA-NO), no se modifica. La inervación sensitiva no participa en esta respuesta ni se modifica con la gestación.
2. En ratas madre tras 21 de lactancia, aumenta la respuesta contráctil inducida por EE. Este incremento está mediado por una mayor liberación de ATP desde las terminaciones simpáticas, mientras que no se modifica ni la liberación ni la respuesta a NA; junto a una disminución en la liberación de NO aunque la respuesta vasodilatadora a DEA-NO es similar. No se observa participación ni alteraciones en la inervación sensitiva.
3. En hipertensión portal inducida por triple ligadura de la vena porta a corto plazo, 1 mes, se observa una disminución en la respuesta vasoconstrictora inducida por EE que está mediada por una diminución en la liberación de NA mientras que su respuesta contráctil no se modifica; y un incremento tanto en la liberación de

NO como del péptido relacionado con el gen de la calcitonina (CGRP), mientras que sus respuestas vasodilatadoras no se modifican. A largo plazo, 21 meses, no se producen ninguna de las alteraciones observadas a corto plazo.

4. En ratas macho adultas, descendientes de madres diabéticas, se incrementa la respuesta contráctil inducida por EE. Este incremento está mediado por un aumento en la liberación de NA y ATP junto a un incremento en la liberación de NO. Las respuestas vasomotoras a NA y DEA-NO no se modifican. La inervación sensitiva no está implicada en este efecto.
5. El ejercicio físico moderado (8 semanas, 5 días/semana, 50 min) revierte la incrementada respuesta vasoconstrictora observada en SHR. En este efecto están implicados una disminución en la liberación de NA y un aumento en su respuesta contráctil; y un incremento en la liberación y biodisponibilidad de NO. La inervación sensitiva participa en la respuesta contráctil aunque esta participación no se modifica con el ejercicio físico.
6. En ratas alimentadas con una dieta rica en grasa (8 semanas), se observa un incremento en la respuesta vasoconstrictora inducida por EE. El ejercicio físico moderado (8 semanas, 5 días/semana, 50 min) realizado de forma simultánea con la dieta previene el incremento observado. Este efecto está mediado por un con una disminución en la liberación de NA junto a incremento en la liberación de NO y ATP, mientras que las respuestas vasomotoras a NA y DEA-NO no se modifican. La inervación sensitiva no participa en ninguna de estas respuestas.
7. La incubación con LPS produjo alteraciones que fueron dependientes del tiempo de exposición. A corto plazo, 2 horas, se incrementa la respuesta vasoconstrictora inducida por EE mediada por a una incrementada respuesta a NA junto con una menor liberación de NO. A largo plazo, 5 horas, disminuye este incremento debido a una restauración en la función simpática y el mantenimiento en la reducción de la función nitrérgica.
8. Los fármacos estabilizadores de células cebadas modifican la respuesta contráctil inducida por EE de forma opuesta. El ketotifen la incrementa a través de una disminución en la liberación de NO y un aumento en la respuesta a DEA-NO, sin modificar la función adrenérgica; mientras que el tranilast la disminuye mediante una diminución en la respuesta a NA junto con una menor liberación de NO.

9. En vasos de resistencia, el tranilast altera la función endotelial incrementando la relajación inducida por acetilcolina (ACh). En este efecto no están implicados el NO ni prostanoides derivados de la ciclooxigenasa (COX), sino a una mayor participación del factor hiperpolarizante derivado de endotelio (EDHF) cuyo efecto está mediado a través de canales de potasio dependientes de calcio de baja conductancia.

La amplitud de resultados obtenidos en esta tesis doctoral nos permite realizar varias consideraciones globales:

- La existencia de inervación nitrérgica funcional en el control del tono vascular en la arteria mesentérica superior de todas las cepas analizadas.
- La inervación sensitiva no participa en la respuesta contráctil inducida por EE en situaciones fisiológicas, mientras que en las distintas situaciones patológicas analizadas sólo contribuye a la vasodilatación esplácnica asociada a la hipertensión portal.
- Los mecanismos por los cuales se incrementa o disminuye la respuesta vasoconstrictora inducida por EE no son homogéneos y difieren en función de la situación experimental analizada.
- El incremento en la respuesta vasoconstrictora inducida por la liberación de los distintos neurotransmisores en arteria mesentérica superior no se ve afectada por la disfunción endotelial.
- No parece existir una interacción entre la inervación nitrérgica y adrenérgica.

Introducción

El sistema cardiovascular está formado por el corazón que impulsa la sangre a través de los vasos sanguíneos a los distintos órganos. Tiene como función principal satisfacer las necesidades nutricionales, metabólicas, hormonales e inmunes de los tejidos manteniendo así, la homeostasis del organismo. Para ello, el corazón bombea la sangre a través de las arterias y ésta retorna por medio de las venas. Aunque la circulación de cada tejido concreto tiene características peculiares, podemos aplicar ciertos principios generales a toda la circulación. Así, la estructura y diámetro de los vasos van a depender de la localización y función que lleven a cabo, se distinguen en:

- Arterias: transportan la sangre a los tejidos bajo presiones elevadas.
- Arteriolas: ramas terminales de las arterias que actúan como válvulas de control del flujo hacia los capilares.
- Capilares: su función es intercambiar sustancias entre la sangre y el líquido intersticial.
- Vénulas: recogen la sangre de los capilares, para ir confluyendo en venas cada vez de mayor calibre, hasta retornar al corazón.

La circulación esplácnica es la parte de la circulación sistémica que irriga la porción abdominal del tubo digestivo, así como el bazo, el páncreas y el hígado. Recibe aproximadamente una cuarta parte del gasto cardíaco, participando en la regulación de la resistencia periférica y consecuentemente de la presión arterial. La contracción o relajación de los vasos de la circulación esplácnica le permite actuar como un sistema de adaptación que garantiza la redistribución de la sangre desde el territorio abdominal hacia la circulación general, con el fin de mantener el flujo sanguíneo adecuado en los órganos vitales. Este mecanismo permite el control de la presión sanguínea sistémica y regulación del flujo sanguíneo tisular (Gourley y Gering, 2005)

Embriológicamente, los vasos mesentéricos tienen su origen en el segmento ventral primitivo, siendo la decimotercera arteria la que da origen a la arteria mesentérica superior (Rosenblum y cols., 1997); la cual forma parte de circulación esplácnica. La arteria mesentérica superior emerge desde la aorta y es la fuente principal de irrigación intestinal.

Características generales de la pared arterial

Es un sistema dinámico, cuyos componentes se modifican o reorganizan en respuesta a estímulos fisiológicos y/o patológicos. En la pared vascular se distinguen tres capas concéntricas: la interna o túnica íntima, la capa media y la externa o túnica adventicia (Figura 1).

1. Túnica íntima

Es la capa interna de la pared arterial y está constituida por un epitelio simple y plano de células endoteliales orientadas longitudinalmente y unidas estrechamente a una lámina basal y tejido conjuntivo subendotelial; está separada de la túnica media por la lámina elástica interna. Las células endoteliales son capaces de detectar cambios físicos, como en el flujo sanguíneo y presión arterial; y químicos como hipoxia. Como respuesta producen múltiples sustancias vasoactivas que actúan de forma exocrina, paracrina y autocrina; y que tienen un papel primordial en la regulación del tono vascular; procesos de inflamación, crecimiento de células vasculares lisas, agregación plaquetaria y coagulación plasmática (Barton y Haudenschild, 2001; Vanhoutte y cols., 2009). Entre estos mediadores vasoactivos, se incluyen vasodilatadores como óxido nítrico (NO), prostaciclina (PGI_2) (Furchgott y Vanhoutte, 1989) y el factor hiperpolarizante derivado de endotelio (EDHF) (Rubanyi 1991). Los principales factores vasoconstrictores liberados por el endotelio son la endotelina 1, prostanoides vasoconstrictores como tromboxano A2 (TXA_2) y prostaglandina E_2 (PGE_2) y especies reactivas de oxígeno (ROS) (Tang y Vanhoutte 2009).

2. Túnica media

Es la capa intermedia de la pared vascular, está formada por capas concéntricas de células musculares lisas entre las que se interponen cantidades variables de matriz extracelular. La túnica media está separada de la túnica adventicia por una segunda capa de fibras elásticas, la lámina elástica externa.

Las fibras musculares lisas de la túnica media son las responsables del mantenimiento de la tensión muscular, regulando el diámetro de la luz del vaso, siendo capaces de

contraerse y relajarse en respuesta a diferentes estímulos. Los componentes de la matriz extracelular, principalmente elastina y colágeno, van a determinan la distensibilidad de la arteria (Guyton 1991).

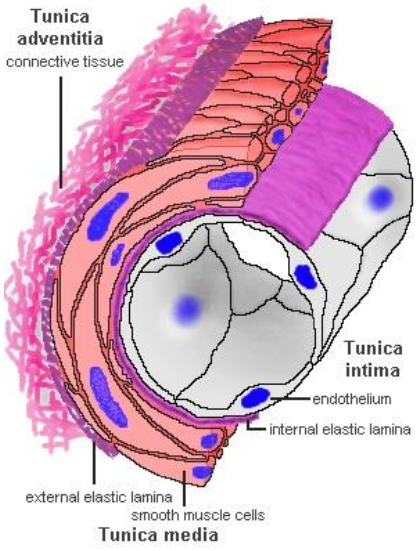

Figura 1: Estructura general de la pared vascular. Tomado de http://www.lab.anhb.uwa.edu.au/mb140/corepages/vascular/vascular.htm

3. Túnica adventicia

Es la capa más externa compuesta por tejido conjuntivo con fibras de colágeno tipo I, y fibras elásticas; diversos tipos celulares como fibroblastos, adipocitos, macrófagos y mastocitos; una red de pequeños vasos sanguíneos que suplen a vasos de mayor tamaño o *vasa vasorum;* (Gutterman 1999; Stenmark y cols., 2013) y terminaciones nerviosas que liberan distintos neurotransmisores (Loesch 2002; Cardinali y Dvorkin, 1999) Históricamente ha sido considerada como una capa poco organizada formada por múltiples tipos celulares. En los últimos años se ha demostrado que tiene una estructura compleja y dinámica que libera sustancias reguladoras tanto de la función como de la estructura vascular (Stenmark y cols., 2013).

El tono vascular está regulado por distintos mecanismos, sustancias vasoactivas liberadas desde las células endoteliales y musculares lisas, además de factores hormonales, metabólicos y neuronales. La respuesta vasomotora neta depende del grado de participación de cada uno de ellos.

Inervación perivascular de la arteria mesentérica

Las terminaciones nerviosas perivasculares liberan neurotransmisores vasoconstrictores y vasodilatadores, que regulan el tono vascular y el flujo sanguíneo. No todas las arterias poseen los mismos tipos de inervación, depende del lecho vascular analizado (Loesch 2002). En la arteria mesentérica se han descrito tres tipos de inervación: simpática, nitrérgica y sensitiva.

1. Inervación simpática

En el sistema nervioso periférico, la rama simpática emerge de la médula espinal torácica y de los dos o tres primeros segmentos lumbares. Estas neuronas preganglionares alcanzan los distintos ganglios paravertebrales y prevertebrales; entre estos últimos se encuentran los ganglios mesentéricos, que dan origen a fibras postganglionares que inervan los órganos de la región abdominal y pelviana así como los vasos mesentéricos (Cardinali y Dvorkin, 1999). Las terminaciones simpáticas liberan noradrenalina (NA) como neurotransmisor principal y los cotransmisores adenosina trifosfato (ATP) y neuropéptido Y (NPY) (Donoso y cols., 1997).

Componente adrenérgico

La NA es una catecolamina que se sintetiza a partir de la tirosina. La enzima tirosina hidroxilasa produce L-DOPA (L-dihidroxifenialanina) a partir de la tirosina, que por medio de la enzima dopadescarboxilasa, se convierte en dopamina. La enzima dopamina β-hidroxilasa, en presencia de oxígeno y ácido ascórbico, transforma en NA la dopamina (Cardinali y Dvorkin, 1999).

La NA se almacena en las vesículas sinápticas y se libera al espacio sináptico por exocitosis. Una vez en la hendidura sináptica, actúa sobre dos tipos de receptores específicos, receptores α y β-adrenérgicos, tanto pre como postsinápticos, que pertenecen a la superfamilia de receptores acoplados a proteína G (Guimaraes y Moura, 2001):

- α1-postsinápticos: en el músculo liso vascular, que activan a la fosfolipasa C (PLC). Esta enzima cataliza la hidrólisis del fosfatidilinositol bifosfato (PIP_2) en inositol trifosfato (IP3) y diacilglicerol (DAG), ambos compuestos actúan como segundos mensajeros provocando la liberación de calcio intracelular y consecuente vasoconstricción (García-Sáinz y cols., 2000).
- α2-presinápticos: se localizan en las terminaciones nerviosas simpáticas (homorreceptores) y no simpáticas (heterorreceptores), su activación inhibe la liberación de NA (Enero y cols., 1972) o modula la liberación de otros neurotransmisores (Marín y Balfagón, 1998), respectivamente.
- β1-postsinápticos: en el músculo liso vascular, que activan a su vez la adenilato ciclasa, produciendo un incremento de adenosín monofosfato cíclico (cAMP), el cual activa la proteína quinasa A (PKA) y provoca la disminución en los niveles de calcio intracelular, originando la relajación del músculo liso vascular (Werstiuk y Lee, 2000). Asimismo se ha descrito la presencia de receptores β-adrenérgicos en endotelio, cuya activación provoca la liberación de óxido nítrico (NO) (Arribas y cols., 1994).
- β2- presinápticos: localizados en las terminaciones nerviosas, a través de los cuales se facilita la liberación de NA (Marín y Balfagón, 1998). La activación de estos receptores en las terminaciones nerviosas nitrérgicas produce un incremento en la liberación de NO (Marín y Balfagón, 1998).

La NA posee mayor afinidad por los receptores α que por los β, y debido al predominio de los receptores α en las células musculares lisas, el efecto vasomotor producido por la NA liberada desde las terminaciones nerviosas adrenérgicas es vasoconstrictor (Vanhoutte 1981), participando en la regulación de la resistencia y el tono vascular.

La acción de NA finaliza por recaptación retrógrada, donde se incorpora a nuevas vesículas sinápticas, y degradación mediante las enzimas monoaminooxidasa (MAO) o catecol-O-metil transferasa (COMT) (Hein 2006).

Componente purinérgico

El cotransmisor purinérgico ATP se sintetiza en las terminaciones nerviosas simpáticas y se almacena en vesículas distintas a las que contienen NA. Una vez liberado actúa sobre receptores purinérgicos tanto pre como postsinápticos (Burnstock y Ralevic, 2013). Se metaboliza por ectonucleotidasas situadas en la superficie de las células

musculares lisas y endoteliales (Zimmermann y cols., 2012) hasta adenosina (Burnstock y Ralevic, 2013).

Los receptores purinérgicos se dividen en dos tipos: P1 y P2. Los P1 son selectivos para adenosina, mientras que los P2 son selectivos para nucleótidos y han sido divididos en P2X y P2Y (Abbracchio y Burnstock, 1994). El efecto vasoconstrictor del ATP en arteria mesentérica procedente de la inervación simpática está mediado por receptores P2X (Ralevic y Burnstock, 1988), y aunque hay evidencias de la expresión de ambos tipos en el músculo liso vascular, no se ha asociado un papel fisiológico a los receptores P2Y. Los receptores P2X son de tipo ionotrópico, su activación por ATP produce una rápida respuesta debida a la entrada de cationes al interior celular produciendo una despolarización de membrana y un flujo de calcio a través de canales de calcio voltaje-dependientes de tipo L (Burnstock y Ralevic, 2013; Rummery y cols., 2007).

Además del efecto vasoconstrictor, la NA y el ATP actúan sinérgicamente disminuyendo la neurotransmisión simpática presinápticamente a través de receptores α2 y P1 (Burnstock 2009).

El cotransmisor NPY tiene un papel menor sobre el músculo liso y actúa fundamentalmente como neuromodulador disminuyendo la liberación de NA y ATP presinápticamente y/o amplificando postsinápticamente las respuestas adrenérgica y purinérgica (Burnstock 2009). Aunque se han estudiado las vías de señalización, sigue sin aclararse totalmente como el NPY contribuye al control vasomotor en condiciones fisiológicas (Westcott y Segal, 2013b).

2. Inervación nitrérgica

La existencia de inervación nitrérgica perivascular desde la que se libera NO y la posibilidad de que tuviera un papel fisiológico o patológico se cuestionó durante mucho tiempo (Toda y Okamura, 2003; Amerini y cols., 1993). Actualmente se ha demostrado su existencia en numerosos lechos vasculares, entre los que se encuentra la arteria mesentérica (Toda y Okamura, 2015).

La síntesis de NO (Figura 2) se lleva a cabo por distintas isoformas de la enzima óxido nítrico sintasa (NOS):

- NOS1 o neuronal (nNOS) está presente en sistema nervioso central y periférico (Ayajiki y cols., 2002), también se ha descrito su expresión en glándula adrenal, en células epiteliales de varios órganos y musculares lisas vasculares (Förstermann y cols., 1994).
- NOS3 o endotelial (eNOS) se expresa principalmente en células endoteliales aunque también ha sido detectada en miocitos cardíacos y plaquetas (Förstermann y cols., 1994), su función está modulada por factores humorales y/o mecánicos (Schulz y cols., 1992; Fisher y cols., 2001).

Entre estas dos isoformas existen numerosas similitudes. Ambas son constitutivas y dependientes de la concentración de calcio para unir calmodulina (CaM). La actividad de ambas enzimas está regulada por procesos de fosforilación mediada por diferentes quinasas y fosfatasas (Rafikov y cols., 2011; Zhou y Zhu, 2009). Contienen múltiples regiones sensibles a fosforilación, aunque las más estudiadas son; para nNOS en la serina 1412 y serina 847 (Zhou y Zhu, 2009) que van a determinar una mayor o menor activación, respectivamente; y para eNOS de forma similar en la serina 1177 y treonina 495 (Rafikov y cols., 2011). Ambas participan, a través de su efecto vasodilatador en el control del tono vascular en situaciones fisiológicas y patológicas, modificando su expresión, activación y acoplamiento.

- NOS2 o inducible (iNOS) fue identificada inicialmente en macrófagos, aunque puede inducirse su expresión en la mayoría de células y tejidos (Förstermann y cols., 1994). Esta isoforma tiene una alta afinidad por la CaM, siendo su unión independiente de las concentraciones de calcio, generando mayores cantidades de NO en situaciones patológicas (Moncada 1997; Schulz y Triggle, 1994).

Al ser estimulada la terminación nerviosa nitrérgica se libera NO, que al ser un gas, difunde a través de las membranas del músculo liso. Está implicado, dado su efecto vasodilatador, en la regulación del tono vascular y la resistencia vascular periférica El NO produce relajación en el músculo liso vascular por la activación de la enzima guanilato ciclasa soluble, incrementando los niveles de guanosín monofosfato cíclico (cGMP) (Toda y Okamura, 2003; Webb 2003). El cGMP activa a la proteína quinasa G (PKG) que activa a su vez a la bomba de calcio (Ca^{2+}-ATPasa) del retículo endoplásmico reduciendo la concentración de calcio intracelular (Yoshida y cols., 1991).

El NO se metaboliza a través de la oxidación producida por especies reactivas de oxígeno (ROS) (Beckman y Koppenol, 1996). Esto implica que la generación de ROS por el metabolismo celular se relaciona estrechamente con el metabolismo de NO y su incremento se ha vinculado con alteraciones en el papel de este neurotransmisor en distintas patologías cardiovasculares (Cai y Harrison, 2000; Wolin 2000). Entre las ROS destacan los aniones superoxido ($O_2^{\cdot -}$), los cuales reaccionan con el NO de manera espontánea e irreversible (Huie y Padmaja, 1993), dando como resultado el anión peroxinitrito ($ONOO^-$) altamente oxidante, que afecta a proteínas, lípidos y ácidos nucleicos (Pacher y cols., 2007).

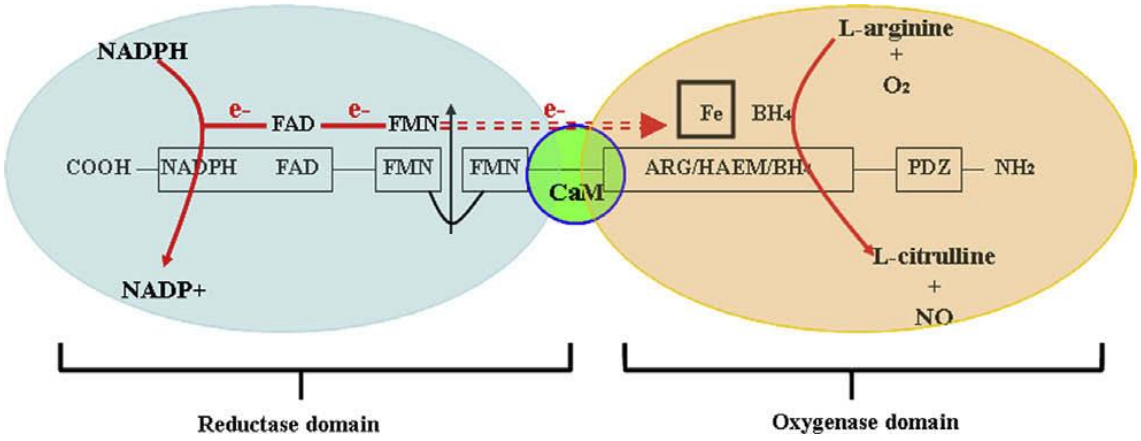

Figura 2. Representación esquemática de la estructura de la óxido nítrico sintasa neuronal y síntesis de NO. Los monónomeros contienen un dominio oxigenasa (N-terminal), una región central de unión a calmodulina (CaM) y un dominio reductasa (C-terminal). El dominio reductasa contiene regiones de unión a nicotinamida adenina dinucleótido fosfato (NADPH), dinucleótido de flavina y adenina (FAD) y mononucleótido de flavina (FMN); y el dominio oxigenasa contiene regiones de unión para L-arginina y tetrahidrobiopterina (BH_4) y un grupo Fe-hemo. Los electrones (e-) son donados por el NADPH reducido y son transferidos desde el dominio reductasa hacia el dominio oxigenasa vía FMN y FAD y se produce la oxidación de la L-arginina en NO y L-citrulina. Adaptado de Zhou y Zhu, 2009.

3. Inervación sensitiva

El núcleo de estas fibras se localiza en los ganglios dorsales de la médula espinal, localizándose las terminaciones nerviosas entre la adventicia y media de los vasos sanguíneos, principalmente arterias (Gulbenkian y cols., 1993; Saetrum Opgaard y

cols., 1995). Liberan diversos neuropéptidos, siendo el principal el péptido relacionado con el gen de la calcitonina (CGRP), liberando también los cotransmisores sustancia P (SP) y neuroquinina A (NKA) pricipalmente (Lundberg y cols., 1985).

El CGRP pertenece a la superfamilia de péptidos de la calcitonina, formada por la calcitonina, adrenomedulina, amilina y CGRP (α y β) (Wimalawansa 1996). Se sintetiza en el soma neuronal y se almacena en vesículas, que lo transportan a través del axón hasta el terminal para liberarlo. Una vez liberado, actúa a través de receptores específicos y su acción finaliza mediante mecanismos de recaptación por los terminales sensitivos (Sams-Nielsen y cols., 2001), metabolismo y transporte hacia el flujo sanguíneo. Estos receptores se componen de tres subunidades: un receptor acoplado a proteína G, calcitonin-like receptor (CL) (Poyner y cols., 2002); una proteína que modifica la actividad del receptor, receptor activity modifying protein 1 (RAMP1) (Poyner y cols., 2002) y otra proteína necesaria para la unión a proteína G y la transducción de la señal, receptor component protein (RCP) (Prado y cols., 2002). La interacción entre el receptor CL y RAMP1 es necesaria para constituir el receptor de CGRP funcional, mientras que otras combinaciones originan receptores para otros péptidos de la misma familia (Kawasaki y cols., 2003).

El CGRP liberado desde las terminaciones nerviosas de la pared vascular participa en la regulación de la resistencia periférica, ejerciendo un efecto vasodilatador. Dicha vasodilatación está mediada por la unión del CGRP a su receptor específico localizado tanto en células endoteliales como células musculares lisas (Wimalawansa 1996). En células del músculo liso vascular la activación del receptor de CGRP activa la enzima adenilato ciclasa, que incrementa los niveles de cAMP y activa a PKA que favorece la apertura de canales de potasio dependientes de ATP (K_{ATP}) y disminuye los niveles de calcio intracelular (Nelson y cols., 1990; Brain y Grant, 2004; Figura 3). Asimismo se ha descrito que el efecto vasodilatador del CGRP sobre músculo liso vascular está mediado por el cGMP (Wimalawansa 1996; Balfagón y cols., 2004). La respuesta dependiente de endotelio está mediada por la producción de NO a través del incremento de cAMP y PKA (Wimalawansa 1996; Brain y Grant, 2004). El NO difunde a la célula muscular lisa donde produce relajación. (Figura 3). Al igual que lo que se observa en otros tipos de terminaciones nerviosas el CGRP regula su propia liberación a través de un mecanismo de retroalimentación negativa, estimulando receptores presinápticos para CGRP (Nuki y cols., 1994).

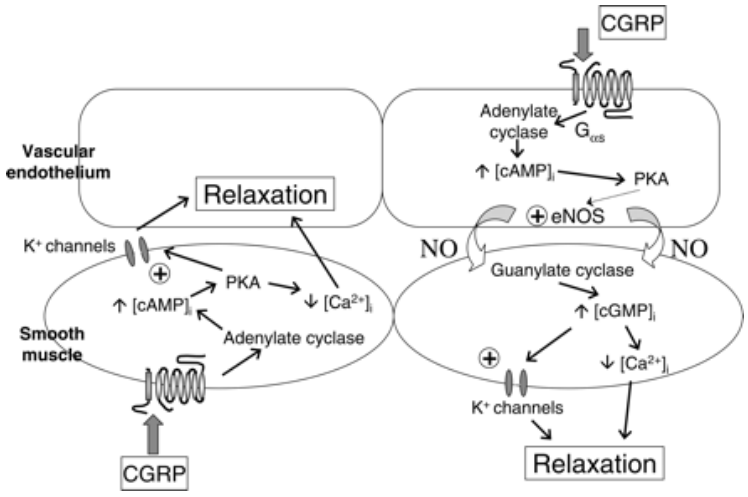

Figura 3: Mecanismos de vasodilatación mediada por CGRP. Izquierda: vasodilatación independiente de endotelio. La activación del receptor de CGRP en la célula muscular lisa está unida a la activación de la enzima adenilato ciclasa y producción de cAMP. El incremento en la concentración de cAMP estimula a la proteína quinasa A (PKA) que activa canales de potasio y disminuye la concentración de calcio intracelular, produciendo la relajación del músculo liso. Derecha: vasodilatación dependiente de endotelio. La activación del receptor de CGRP produce un incremento en la producción de NO por la activación de la óxido nítrico sintasa endotelial (eNOS) mediada por el incremento en cAMP y PKA. El NO difunde a las células del músculo liso donde activa a la guanilato ciclasa produciendo relajación. Adaptado de Brain y Grant, 2004.

Antecedentes

Resultados previos observados en nuestro laboratorio han demostrado que la participación de los distintos tipos de inervación perivascular mesentérica se modifica en situaciones tanto fisiológicas como patológicas.

Las modificaciones de los distintos tipos de inervación están implicadas en la génesis y mantenimiento de las alteraciones vasculares asociadas a múltiples situaciones fisiopatológicas, como envejecimiento o hipertensión arterial. Estas modificaciones pueden ser debidas tanto a cambios en la liberación de los neurotransmisores correspondientes, como a alteraciones en la respuesta de las células del músculo liso vascular a dichos neurotransmisores.

Hipertensión

El incremento del tono vascular observado en hipertensión se ha asociado con disfunción endotelial, estrés oxidativo y remodelado de la pared vascular, entre otros

(Montezano y cols., 2015). La disfunción endotelial se ha vinculado a alteraciones en la formación de NO endotelial y/o mayor metabolismo por ROS, así como una mayor producción y respuesta a sustancias vasoconstrictoras (Paravicini y Touyz, 2006; Félétou y cols., 2010).

La participación de la inervación adrenérgica en el desarrollo de la hipertensión ha sido extensamente estudiada, se ha demostrado que se produce un incremento en la actividad simpática así como alteraciones tanto en la regulación presináptica de la liberación como en los mecanismos de recaptación de la NA (Westfall y cols., 2013). Sin embargo se han descrito resultados contradictorios en cuanto a la respuesta vasoconstrictora a NA (Marín 1993; Tabernero y cols., 1996; Dickhout y Lee, 1997), las diferencias parecen deberse a las alteraciones en la estructura de la pared vascular observada en hipertensión.

Por otra parte, son escasos los trabajos sobre el posible papel de la inervación nitrérgica en el desarrollo de hipertensión, con resultados controvertidos. Se ha descrito tanto una disminución (Quadri y cols., 1999; Hauser y cols., 2005) como un incremento (Piech y cols., 2003; Hojna y cols., 2007) en la expresión de nNOS en sistema nervioso central. Resultados similares han sido observados a nivel periférico y en diferentes lechos vasculares, incluido el mesentérico (Rabelo y cols., 2001; Ferrer y cols., 2003).

En nuestro laboratorio hemos observado que en ratas macho espontáneamente hipertensas (SHR) se produce un incremento en la liberación de NA y una disminución en la respuesta vasoconstrictora a dicho neurotransmisor, así como un incremento en la participación de la inervación nitrérgica caracterizada por un aumento en la liberación de NO, mediada por la mayor actividad de la proteína quinasa C (PKC), y en la respuesta vasodilatadora a donantes de NO (Marín y cols., 2000). Adicionalmente hemos observado en ratas macho SHR que la aldosterona incrementa la expresión de la subunidad RAMP1 del receptor de CGRP, mecanismo por el cual incrementa la respuesta vasodilatadora a CGRP (Balfagón y cols., 2004; Márquez-Rodas y cols., 2006). Por otra parte, en el modelo de hipertensión inducido por ouabaína, la función de la inervación adrenérgica no está alterada, mientras que se incrementa la liberación de NO mediada por la mayor expresión de nNOS y no se modifica la respuesta vasodilatadora a nitroprusiato sódico (SNP) (Xavier y cols., 2004). En SHR hembras, sin embargo, la función adrenérgica no se modifica, mientras que se disminuye la

liberación de NO, mediada por una menor expresión de nNOS; y se incrementa tanto la liberación de CGRP como su respuesta vasodilatadora (del Campo y cols., 2004).

Estos resultados obtenidos en machos sugieren que las alteraciones en la inervación adrenérgica en hipertensión difieren dependiendo del modelo experimental utilizado, mientras que las modificaciones en la función de la inervación nitrérgica son similares, un incremento en su participación, sugiriendo que actúa como un mecanismo compensador.

Sin embargo en hembras observamos el proceso contrario en cuanto a la liberación de NO, disminuye mientras que no se modifica la función adrenérgica, indicando la existencia de dimorfismo sexual en las alteraciones de la inervación perivascular de la arteria mesentérica en hipertensión.

Diabetes

La diabetes es una enfermedad metabólica caracterizada por hiperglucemia, en la que se observan diferentes alteraciones vasculares, que son la causa más común de morbilidad y mortalidad. Se ha demostrado el desarrollo de disfunción endotelial tanto en pacientes como en modelos animales (Johnstone y cols., 1993; Pieper 1999) lo cual se ha sugerido que constituye el origen de estas alteraciones en diferentes lechos vasculares (Bertoluci y cols., 2015). La disfunción endotelial no es un proceso homogéneo en sus características y distribución, sino que varía con la duración de la exposición a la hiperglucemia y el lecho vascular analizado (Davel y cols., 2011), lo cual facilita la progresión de las alteraciones vasculares en diabetes. La característica más notable de la disfunción endotelial en diabetes es la disminución de la liberación y/o biodisponibilidad del NO, lo cual se ha asociado tanto al incremento del estrés oxidativo como al déficit de sustrato y/o cofactores cruciales para la actividad de eNOS (Akamine y cols., 2006) aunque también se ha relacionado con la liberación de diferentes sustancias vasoconstrictoras como prostanoides derivados de la ciclooxigenasa (COX) (Bagi y cols., 2006).

A pesar del control de los niveles de glucosa en pacientes diabéticos, éstos mantienen un alto riesgo de sufrir enfermedades cardiovasculares, siendo las neuropatías una de las mayores complicaciones en diabetes. Datos epidemiológicos indican que el 50% de los pacientes diabéticos sufren alteraciones en la función neuronal (Boulton y cols., 2005).

Con respecto a las posibles alteraciones en la inervación simpática se ha descrito que no existen modificaciones (Weber y MacLeod, 1994; Speirs y cols., 2006) y una reducción (Hart y cols., 1988; Ralevic y cols., 1995) en la vasoconstricción inducida por EE. Estas diferencias en los resultados se han asociado a los distintos tiempos de exposición a la hiperglucemia y al lecho vascular analizado (Johansen y col., 2013).

Sobre la posible alteración de la inervación nitrérgica los datos son escasos, salvo en la disfunción eréctil en la cual la disminución de su función se asocia tanto a una menor liberación de NO como a un incremento de su metabolismo. Estudios recientes asocian esa disfunción al desacoplamiento de nNOS en arterias peneanas mediado por una disminución de l-arginina y/o del cofactor tetrahidrobiopterina (BH_4) (Sánchez y cols., 2012). Al igual que en la disfunción endotelial, diversos trabajos han sugerido que la evolución de esta neuropatía es dependiente del tiempo (Cellek y cols., 2003). Los resultados obtenidos en nuestro laboratorio en un modelo de diabetes inducido por estreptozotocina muestran en arteria mesentérica un incremento en la liberación de NO neuronal mediado por la activación de PKC; asimismo se ha observado una mayor función simpática mediada por un incremento en la liberación de NA (Ferrer y cols., 2000). Siendo el resultado neto un incremento en la respuesta vasoconstrictora a la EE. Estos datos tomados en su conjunto indican que en diabetes se producen alteraciones en la función de la inervación perivascular y que estas alteraciones dependen del lecho vascular analizado y del tiempo de evolución.

Cirrosis

La cirrosis hepática es una patología frecuente que se sitúa dentro de las 10 causas más comunes de muerte en los países desarrollados (Stewart y Day, 2003). Esta patología cursa con el desarrollo de hipertensión portal y circulación esplácnica hiperdinámica, cuyos efectos acaban afectando tanto a la circulación hepática y esplácnica, como a la circulación cardiaca, pulmonar, renal y cerebral (Lockwood y cols., 1991; Palma y Fallon, 2006). Los mecanismos fisiopatológicos implicados en estos cambios cardiovasculares son complejos, ya que se ha descrito la participación de factores origen neurogénico, humoral y endotelial (Al-Hamoudi 2010; Xavier y cols., 2010), cuyas modificaciones difieren en función del lecho vascular analizado, así se observa una intensa vasoconstricción intrahepática y una marcada vasodilatación esplácnica.

En el lecho esplácnico se ha descrito un incremento en la liberación y sensibilidad de factores vasodilatadores como NO (Morales-Ruiz y cols., 1996; Iwakiri 2007) y PGI_2 (Vaughan y cols., 2005); así como una disminución en la respuesta a factores vasoconstrictores como NA (Battaglia y cols., 2006) y angiotensina II (Hennenberg y col., 2009), induciendo alteraciones hemodinámicas que agravan y prolongan la enfermedad e incluso ser causa de muerte (Groszmann 1994).

En nuestro laboratorio, en un modelo de cirrosis inducido por administración oral de tetracloruro de carbono, los resultados obtenidos en arteria mesentérica superior confirman la diminución en la función simpática, mediada tanto por una menor liberación como respuesta a NA, y el incremento en la función nitrérgica mediada por un aumento en la expresión de nNOS y liberación de NO (datos no publicados). Adicionalmente, se observa un incremento en la liberación de CGRP y de su respuesta vasodilatadora debida a un incremento en la apertura de canales K_{ATP}, jugando, por tanto, un papel en la vasodilatación esplácnica observada en cirrosis (Blanco-Rivero y cols., 2011).

Además de la valoración de las alteraciones en la función de la inervación perivascular mesentérica en situaciones patológicas, en nuestro grupo también se han analizado posibles modificaciones en situaciones fisiológicas.

Envejecimiento

El envejecimiento se asocia con cambios morfológicos y funcionales en distintos sistemas, incluido el sistema cardiovascular, donde la incidencia de distintas patologías se encuentra incrementada.

Entre los factores implicados en el incremento de riesgo cardiovascular asociado a la edad, destacan la disfunción endotelial (Seals y cols., 2011) y el remodelado vascular (Barodka y cols., 2011). En modelos animales se ha descrito un aumento, disminución y no modificación de la expresión/activación de eNOS (Durrant y cols., 2009; van der Loo y cols., 2000; Rippe y cols., 2010), asimismo se ha implicado el incremento del estrés oxidativo (Lesniewski y cols., 2009; Durrant y cols., 2009) y disminución de la capacidad antioxidante (Wang y Shah, 2015) así como a una menor respuesta a prostaglandinas vasodilatadoras (Singh y cols., 2002).

La actividad del sistema renina-angiotensina y el incremento en la actividad nerviosa simpática, producen alteraciones en la proliferación de células musculares lisas (Wang y Shah, 2015; Dinenno y cols., 2000), participando en el desarrollo del remodelado vascular y en la respuesta vascular a vasoconstrictores. Los datos con respecto a la respuesta a sustancias vasoconstrictoras son contradictorios, con disminución (Seals y Dinenno, 2004) y aumento (Dinenno y cols., 2000).

El papel de la inervación perivascular en los cambios vasculares asociados a la edad es escasamente conocido. En nuestro laboratorio, en arteria mesentérica de ratas con 22-24 meses se ha observado una mayor respuesta vasoconstrictora a NA, mientras que la liberación no se modifica. En el caso de la inervación nitrérgica, se produce una disminución en la función, debido a una menor liberación de NO sin alteraciones en el metabolismo y sensibilidad del músculo al NO (Ferrer y Balfagón, 2001). Estudios recientes han descrito una menor función de los nervios sensitivos en el lecho mesentérico (Westcott y Segal, 2013a), sin embargo en nuestro modelo la inervación sensitiva no parece estar implicada en las alteraciones asociadas con la edad.

Estos resultados, en su conjunto, indican que la inervación adrenérgica juega un papel relevante en el mantenimiento del tono vasomotor de la arteria mesentérica, y que se altera su función en diversas situaciones fisiopatológicas, participando en el desarrollo y/o mantenimiento de las mismas. Estos datos cuestionan la idea de que la inervación nitrérgica, aunque exista no tiene un papel en el control vascular y de que sólo el NO de origen endotelial participa en la regulación del tono vascular periférico como han sugerido algunos autores (Toda y Okamura, 2003; Amerini y cols., 1993) y refuerzan al NO procedente de la inervación nitrérgica como un elemento más en la regulación del tono vascular. Este importante papel de la inervación nitrérgica se ha puesto de manifiesto en humanos donde el inhibidor específico de la nNOS, S-metil-L-tiocitrulina, redujo el flujo sanguíneo basal en el antebrazo y en la circulación coronaria. (Melikian y col., 2009).

Objetivos

Estos resultados, tomados en su conjunto, nos indican que la función de la inervación perivascular en la arteria mesentérica superior se modifica tanto en situaciones fisiológicas como patológicas y que los mecanismos implicados tanto en el incremento como en la disminución de la respuesta inducida por estimulación eléctrica varían según el modelo experimental.

Se ha descrito que el flujo de la circulación esplácnica se modifica o altera en distintas situaciones. En la gestación y la lactancia materna, con el fin de adaptarse a las nuevas demandas del organismo (Melchiorre y cols., 2012; Svennersten-Sjaunja y Olsson, 2005); en la hipertensión portal se produce el síndrome de circulación hiperdinámica asociada a una gran vasodilatación en este lecho (Bosch y cols., 2015) y durante la sepsis y shock séptico asociada a una disminución en la resistencia vascular y una anómala redistribución del flujo sanguíneo (Landry y Oliver, 2001). Los trabajos que han abordado el estudio de los mecanismos implicados en estas modificaciones por diversos grupos de investigación se han centrado preferentemente en el estudio de las alteraciones de la función endotelial (Magness y cols., 1996; Levy y cols., 2010; Iwakiri 2014) siendo menos conocido el papel de los distintos neurotransmisores. Aunque se ha sugerido la participación de la inervación en situaciones fisiológicas y patológicas como gestación (van Drongelen y cols., 2012) e hipertensión portal (Iwakiri 2014), es un aspecto prácticamente inexplorado.

Asimismo, se ha observado que la descendencia macho adulta de madres diabéticas desarrolla hipertensión. Se ha descrito que alteraciones tanto en la función endotelial (Ramos-Alves y cols., 2012a, 2012b) en vasos mesentéricos como en la función renal (Rocha y cols., 2005) están implicados en este proceso. Previamente ya hemos descrito las alteraciones en la función perivascular en diversos modelos de hipertensión (Marín y cols., 2000; Márquez-Rodas y cols., 2006; Xavier y cols., 2004). Consecuentemente, en este modelo de programación fetal podrían producirse alteraciones en la función de la inervación perivascular.

El sedentarismo constituye un factor de riesgo en el desarrollo de enfermedades cardiovasculares. La práctica regular de ejercicio físico ha demostrado tener efectos beneficiosos en el sistema cardiovascular (Belardinelli y cols., 1999; Phillips y cols., 2015). Se ha descrito que el ejercicio físico influye sobre la función de la inervación

simpática (Leosco y cols., 2013), sin embargo no son conocidos sus efectos sobre la inervación nitrérgica y sensitiva.

Se ha demostrado la existencia de comunicación entre neuronas del tracto gastrointestinal y células cebadas de la cavidad abdominal (Van Nassauw y cols., 2007). La localización de los mastocitos o células cebadas en la túnica adventicia sugiere una interacción entre éstas y la inervación perivascular. Por lo tanto, la activación o estabilización de las células cebadas pueden afectar a la función de esta inervación y del endotelio vascular.

Por lo tanto, nuestro objetivo ha sido analizar la implicación de las modificaciones/alteraciones en la función de la inervación perivascular simpática, nitrérgica y sensitiva de la arteria mesentérica superior en:

1. Gestación y Lactancia
2. Hipertensión Portal
3. Descendencia adulta de madres diabéticas
4. Efecto del LPS sobre la función neuronal.
5. Efectos del ejercicio físico sobre la función neuronal en hipertensión y obesidad inducida por una dieta rica en grasa
6. Efecto de los estabilizadores de células cebadas, ketotifen y tranilast, en arteria mesentérica superior.
7. Efecto del tranilast sobre la función endotelial de arteria mesentérica de resistencia.

Resultados

Artículo 1: Alterations in perivascular sympathetic and nitrergic innervation function induced by late pregnancy in rat mesenteric arteries

Sastre E, Blanco-Rivero J, Caracuel L, Callejo M, Balfagón G.

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RESEARCH ARTICLE

Alterations in Perivascular Sympathetic and Nitrergic Innervation Function Induced by Late Pregnancy in Rat Mesenteric Arteries

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Abstract

Background and Purpose

We investigated whether pregnancy was associated with changed function in components of perivascular mesenteric innervation and the mechanism/s involved.

Experimental Approach

We used superior mesenteric arteries from female Sprague-Dawley rats divided into two groups: control rats (in oestrous phase) and pregnant rats (20 days of pregnancy). Modifications in the vasoconstrictor response to electrical field stimulation (EFS) were analysed in the presence/absence of phentolamine (alpha-adrenoceptor antagonist) or L-NAME (nitric oxide synthase-NOS- non-specific inhibitor). Vasomotor responses to noradrenaline (NA), and to NO donor DEA-NO were studied, NA and NO release measured and neuronal NOS (nNOS) expression/activation analysed.

Key Results

EFS induced a lower frequency-dependent contraction in pregnant than in control rats. Phentolamine decreased EFS-induced vasoconstriction in segments from both experimental groups, but to a greater extent in control rats. EFS-induced vasoconstriction was increased by L-NAME in arteries from both experimental groups. This increase was greater in segments from pregnant rats. Pregnancy decreased NA release while increasing NO release. nNOS expression was not modified but nNOS activation was increased by pregnancy. Pregnancy decreased NA-induced vasoconstriction response and did not modify DEA-NO-induced vasodilation response.

Conclusions and Implications

Neural control of mesenteric vasomotor tone was altered by pregnancy. Diminished sympathetic and enhanced nitrergic components both contributed to the decreased vasoconstriction response to EFS during pregnancy. All these changes indicate the selective participation of sympathetic and nitrergic innervations in vascular adaptations produced during pregnancy.

Introduction

Pregnancy is associated with a decrease in systemic vascular resistance that, despite the marked increase in blood volume and cardiac output, maintains or reduces maternal blood pressure, in both experimental animals and humans. Adaptations to pregnancy have been studied in several vascular beds, but the mechanisms underlying the altered vessel function are complex and only partially understood.

Vascular adaptations to pregnancy include both an endothelium-dependent pathway associated with increased production of vasodilators [1] and an endothelium-independent pathway associated with altered vasomotor smooth muscle cell responses to different vasoactive substances [2,3,4], that decrease myogenic reactivity [5] and increase vascular compliance [6]. However, activation of additional endothelium-independent pathways has been strongly suggested [7]. Perivascular innervation has a significant influence on peripheral vascular resistance involving the sympathetic, cholinergic, nitrergic, peptidergic and/or sensory innervations, which are specific to the vascular bed under consideration.

The mesenteric artery plays a pivotal role in global peripheral resistance in rats, especially in pregnancy; during this physiological process, mesenteric perfusion is strongly increased. These arteries are innervated by sympathetic nerves, which mediate vasoconstriction mainly via noradrenaline (NA) release, but also by nitrergic innervation, which induces vasodilatation by nitric oxide (NO) release, and sensory innervation through release of the vasodilator calcitonin gene-related peptide neuropeptide (CGRP) [8,9,10]. Electric field stimulation (EFS) produces a vasomotor response that is the integrated result of the effect of these different neurotransmitters [10]. The alterations in the functional roles of these components have been associated with changes in synthesis, release, response and/or metabolism of the different neurotransmitters in several physiological and pathological circumstances [11,12,13,14].

Neuronal adaptation to pregnancy by mesenteric arteries it has been reported to be time-dependent. In late pregnancy diminished sympathetic nerve-mediated constriction has been associated with a decreased vasoconstrictor response to NA [7], while possible changes in NA release have been suggested but not investigated [4]. No changes have been reported in sensory innervation [4] but there is an increased vasodilation to CGRP [7,15,16]. It is widely known that estrogens modulate vascular tone activating endothelial nitric oxide synthase (eNOS) and several studies have reported that vascular adaptation in pregnancy is associated with an increase in eNOS protein expression [17,18,19]. In previous studies we have observed that changes in levels of sex steroids are associated with changes in nitrergic innervation function [14,20]. However, to the best of our knowledge, the possible role of nitrergic innervation in vascular adaptations to pregnancy remains unexplored.

Taking these data into account, we considered it relevant to study possible simultaneous changes in the different kinds of perivascular innervation during pregnancy, consequently the aim of this work is to analyze whether the possible functional changes in sympathetic, nitrergic

and sensory innervations in late pregnancy could be associated with the decreased vascular resistance observed in the mesenteric artery, as well as the mechanisms that may be implicated.

Materials and Methods

Animals

Female Sprague-Dawley rats (4–6 months old) were obtained from the Animal Quarters and housed in the Animal Facility of the Universidad Autónoma de Madrid (registration number EX-021U) in accordance with guidelines 609/86 of the E.E.C., R.D. 233/88 of the Ministerio de Agricultura, Pesca y Alimentación of Spain, and the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institute of Health [NIH publication No. 85–23, revised 1996]. All experimental procedures involving animal use were approved by the Ethics Committee of the Universidad Autónoma de Madrid. Rats were housed at a constant room temperature, humidity, and light cycle (12:12 h light-dark) with free access to tap water and fed with standard rat chow *ad libitum*, and were divided into two groups: Control (virgin females in oestrous phase) and pregnant rats (20 days of pregnancy). The stage of the oestrous cycle was determined by examination of vaginal smears; oestrous females exhibited the presence of cornified cells. The day when a vaginal plug was found was considered to be day 1 of pregnancy.

Animals were sacrificed by CO_2 inhalation followed by decapitation; superior mesenteric artery was carefully dissected, cleaned of connective tissue and placed in Krebs-Henseleit solution (KHS, in mmol/L: NaCl 115, $CaCl_2$ 2.5, KCl 4.6, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 25; glucose 11.1; Na_2EDTA 0.03) at 4°C. For protein expression analysis, some segments were rapidly frozen in liquid nitrogen and kept at -80°C until the day of analysis.

Vascular reactivity

The method used for isometric tension recording has been described in full elsewhere [10,21]. Two parallel stainless steel pins were introduced through the lumen of the vascular segment: one was fixed to the bath wall, and the other connected to a force transducer (Grass FTO3C; Quincy, MA, USA); this, in turn, was connected to a model 7D Grass polygraph. For EFS experiments, segments were mounted between two platinum electrodes 0.5 cm apart and connected to a stimulator (Grass, model S44) modified to supply adequate current strength. Segments were suspended in an organ bath containing 5 mL of KHS at 37°C and continuously bubbled with a 95% O_2 to 5% CO_2 mixture (pH of 7.4). Some experiments were performed in endothelium-denuded segments to eliminate the main source of vasoactive substances, including endothelial NO. This avoided possible actions by different drugs on endothelial cells that could lead to misinterpretation of results. Endothelium was removed by gently rubbing the luminal surface of the segments with a thin wooden stick. The segments were subjected to a tension of 0.5 g, which was readjusted every 15 min during a 90-min equilibration period before drug administration. After this, vessels were exposed to 75 mmol/L KCl to check their functional integrity. After a washout period, the presence/absence of vascular endothelium was tested by the ability/inability of 10 μmol/L acetylcholine (ACh) to relax segments precontracted with NA (1 μmol/L).

Vasodilator response to ACh (0.1 nmol/L- 10 μmol/L) was tested in endothelium-intact arteries precontracted with NA (1μmol/L) from all experimental groups.

Frequency-response curves to EFS (1, 2, 4 and 8 Hz) were performed in endothelium-intact and endothelium-denuded mesenteric segments from all experimental groups. The parameters used for EFS were 200 mA, 0.3 ms, 1–8 Hz, for 30 s with an interval of 1 min between each stimulus [10], the time required to recover basal tone. Two successive frequency-response curves separated by 1-hour intervals produced similar contractile responses. To evaluate the

neural origin of the EFS-induced contractile response, the nerve impulse propagation blocker, tetrodotoxin (TTX, 0.1 μmol/L) was added to the bath 30 min before the second frequency-response curve was performed.

To determine the participation of the adrenergic component of sympathetic innervation in EFS-induced responses in endothelium-denuded segments from control and pregnant rats, 1 μmol/L phentolamine, an alpha-adrenoceptor antagonist was added to the bath 30 min before performing the frequency-response curve. Additionally, the vasoconstrictor response to exogenous NA (1 nmol/L- 10 μmol/L) was tested in segments from both experimental groups.

The method to deplete sympathetic innervation has been used previously by our group in this artery [22]. Briefly, endothelium-denuded mesenteric segments from control and pregnant rats were incubated at room temperature for 10 minutes in KHS (NaHCO3 and NaH2PO4 were omitted, unbuffered solution) containing 0.02 mmol/L glutathione and 1.46 mmol/L of the neurotoxin 6-hydroxydopamine (6-OHDA). The pH of this solution was adjusted to 4.9 with 0.05 mmol/L NaOH and then the solution was covered with paraffin oil. Subsequently, the arteries were immersed in normal KHS and EFS-induced contraction experiments were performed. After EFS-contraction experiments mesenteric segments from control and pregnant rats were exposed to 75 mmol/L KCl to check that their functional integrity was not affected by 6-OHDA.

To study the possible participation of sensory innervation in EFS-induced responses in endothelium-denuded segments from control and pregnant rats, 0.5 μmol/L CGRP (8–37), a CGRP receptor antagonist was added to the bath 30 min before performing the second frequency-response curve.

To analyse the participation of NO in the EFS-induced response in endothelium-denuded segments from control and pregnant rats, 0.1 mmol/L N^{ω}-nitro-L-arginine methyl ester (L-NAME), a non-specific inhibitor of nitric oxide synthase (NOS), was added to the bath 30 min before performing the second frequency-response curve. The vasodilator response to the NO donor, diethylamine NONOate (DEA-NO, 0.1 nmol/L-0.1 mmol/L) was determined in NA-precontracted arteries from both experimental groups. To assess the participation of superoxide anion in DEA-NO-induced vasodilation, 0.1 mmol/L tempol, a superoxide anion scavenger, was added to the bath before the frequency-response curve to DEA-NO was performed.

Noradrenaline release

Endothelium-denuded mesenteric segments from control and pregnant rats were preincubated for 30 min in 5 mL of KHS at 37°C and continuously gassed with a 95% O_2-5% CO_2 mixture (stabilisation period). This was followed by two washout periods of 10 min in a bath of 0.4 mL of KHS. Then, the medium was collected to measure basal release. Next, the organ bath was refilled, and cumulative EFS periods of 30 s at 1, 2, 4 and 8 Hz were applied at 1 min intervals. Afterwards, the medium was collected to measure EFS-induced neurotransmitter release. The EFS-induced NA release was calculated by subtracting basal NA release from that evoked by EFS. Mesenteric segments were weighed in order to normalise the results.

NA release was measured using the Noradrenaline Research EIA (Labor Diagnostica Nord, Gmbh and Co., KG, Nordhon, Germany). The assay was performed following the manufacturer's instructions. Results were expressed as ng NA/mL mg tissue.

Nitric Oxide release

NO release was measured using fluorescence emitted by the fluorescent probe 4,5-diaminofluorescein (DAF-2) [23]. Endothelium-denuded mesenteric arteries from control and pregnant rats were subjected to a 60-minute equilibration period in HEPES buffer (in mmol/L:

NaCl 119; HEPES 20; $CaCl_2$ 1.2; KCl 4.6; $MgSO_4$ 1; KH_2PO_4 0.4; $NaHCO_3$ 5; glucose 5.5; Na_2HPO_4 0.15; pH 7.4) at 37°C. Arteries were incubated with 2 μmol/L DAF-2 for 30 min. The medium was then collected to measure basal NO release. Once the organ bath was refilled, cumulative EFS periods of 30 s at 1, 2, 4 and 8 Hz were applied at 1 min intervals. Afterwards, the medium was collected to measure EFS-induced NO release. The fluorescence of the medium was measured at room temperature using a spectrofluorometer (LS50 Perkin Elmer Instruments, FL WINLAB Software, Whaltmann, MA, USA) with excitation wavelength set at 492 nm and emission wavelength at 515 nm. Mesenteric segments were weighed in order to normalize the results.

The EFS-induced NO release was calculated by subtracting basal NO release from that evoked by EFS. Also, blank samples were collected in the same way from segment-free medium in order to subtract the background emission. Some assays were performed in the presence of 0.1 mmol/L L-NAME, 0.1 mmol 7-nitroindazol (7-NI), the specific nNOS inhibitor, 1 μmol/L 1400W, the specific iNOS inhibitor, or 0.1 μmol/L TTX. The amount of NO released was expressed as arbitrary units/mg tissue.

Detection of $O_2^{\cdot -}$

$O_2^{\cdot -}$ levels were measured using lucigenin chemiluminescence [24]. Endothelium-denuded mesenteric segments from control and pregnant rats were rinsed in KHS for 30 min, equilibrated for 30 min in HEPES buffer at 37°C, transferred to test tubes that contained 1 mL HEPES buffer (pH 7.4) with lucigenin (5 μmol/L) and then kept at 37°C. The luminometer was set to report arbitrary units of emitted light; repeated measurements were collected for 5 min at 10 s intervals and averaged. 4,5-Dihydroxy-1,3-benzene-disulphonic acid "Tiron" (10 mmol/L), a cell-permeant, non-enzymatic $O_2^{\cdot -}$scavenger, was added to quench the $O_2^{\cdot -}$dependent chemiluminescence. Some segments were preincubated with 0.3 mmol/L apocinin, a NADPH oxidase inhibitor, or 0.1 mmol/L allopurinol, a xanthine oxidase inhibitor. Also, blank samples were collected in the same way without mesenteric segments to subtract background emission.

nNOS and P-nNOS Expression

Western blot analysis of nNOS and phosphorylated nNOS (P-nNOS) expression was performed in endothelium-denuded mesenteric segments from control and pregnant rats, as previously described [23,24]. For these experiments, we used mouse monoclonal nNOS antibody (1:1000, Abcam, Cambridge, UK), rabbit polyclonal phospho1417-nNOS antibody (1:1000, Abcam, Cambridge, UK), and monoclonal anti-β-actin-peroxidase antibody (1:50000, Sigma-Aldrich, Spain). Rat brain homogenates were used as a positive control.

Drugs used

L-NA hydrochloride, ACh chloride, diethylamine NONOate diethylammonium salt, CGRP (8–37), TTX, L-NAME hydrochloride, 7-NI, 1400W, phentolamine, apocinin, allopurinol, lucigenin, tiron, tempol and DAF-2 (Sigma-Aldrich, Madrid, Spain) were used. Stock solutions (10 mmol/L) of drugs were made in distilled water, except for NA, which was dissolved in a NaCl (0.9%)-ascorbic acid (0.01% w/v) solution. These solutions were kept at -20°C and appropriate dilutions were made in KHS on the day of the experiment.

Data analysis

The responses elicited by EFS and NA were expressed as a percentage of the initial contraction elicited by 75 mmol/L KCl for comparison between control and pregnant rats. The relaxation

induced by ACh or DEA-NO was expressed as a percentage of the initial contraction elicited by NA (Control: 1373 ± 178.4 mg; pregnant: 1366 ± 126.5 mg; $P>0.05$). For concentration-response curves, non-linear regression and Emax and—log EC50 were performed. Results are given as mean ± S.E.M. Statistical analysis was done by comparing the curve obtained in the presence of the different substances with the previous or control curve by means of repeated measure analysis of variance (ANOVA) followed by the Bonferroni *post-hoc* test using GraphPad Prism 5.0 software (CA, USA). Some results were expressed as differences of area under the curve (dAUC) for EFS obtained in segments from control and pregnant rats. AUC were calculated from the individual concentration-response plots. For dAUC, NO, $O_2^{\cdot-}$ and NA release data, the statistical analysis was done using one-way ANOVA followed by Newman-Keuls' *post-hoc* test. $P<0.05$ was considered significant.

Results

Vasomotor response to KCl

In endothelium-intact mesenteric segments, the vasoconstrictor response to 75 mmol/L KCl was similar in mesenteric segments from control and pregnant rats (Control: 1421 ± 114 mg; pregnant; 1538 ± 67.3 mg; $P>0.05$). Endothelium removal did not alter KCl-induced vasoconstriction (Control: 1677 ± 63.5 mg; pregnant: 1788 ± 54 mg; $P>0.05$).

Vasodilator response to ACh

Vasodilator response to ACh was similar in NA-precontracted segments from both experimental groups (Fig 1, Table 1).

Vascular responses to EFS

The application of EFS induced a frequency-dependent contractile response in both endothelium-intact and endothelium-denuded mesenteric segments from both experimental groups. This vasoconstriction was lower in segments from pregnant rats compared to control animals (Fig 2A and 2B). Endothelium removal increased the EFS-induced contractile response to a similar extent in segments from control and pregnant rats (Fig 2A and 2B, Table 2). EFS-induced contractions were practically abolished by preincubation with neurotoxin TTX (0.1 μmol/L), indicating the neuronal origin of the factors inducing this response (Table 3).

Effect of pregnancy on the sympathetic component of vascular responses to EFS in endothelium-denuded mesenteric segments

Preincubation with the alpha-adrenergic antagonist phentolamine (1 μmol/L) decreased the vasoconstrictor response induced by EFS in segments from both experimental groups (Fig 3A and 3B). This decrease was lower in arteries from pregnant rats (Table 4). The phentolamine-resistant contractile response to EFS was lower in segments from pregnant rats (Table 5). Preincubation with 6-OHDA practically abolished the EFS-induced contraction in segments from control and pregnant rats (Table 3). After 6-OHDA preincubation, vasomotor response to KCl was similar in mesenteric segments from both experimental groups (Control: 1501 ± 95.4 mg; pregnant; 1622 ± 109 mg; $P>0.05$).

The contractile response elicited by exogenous NA (1 nmol/L-10 μmol/L) was reduced in segments from pregnant rats (Fig 3C, Table 1). Additionally, EFS-induced NA release was lower in segments from pregnant rats (Fig 3D).

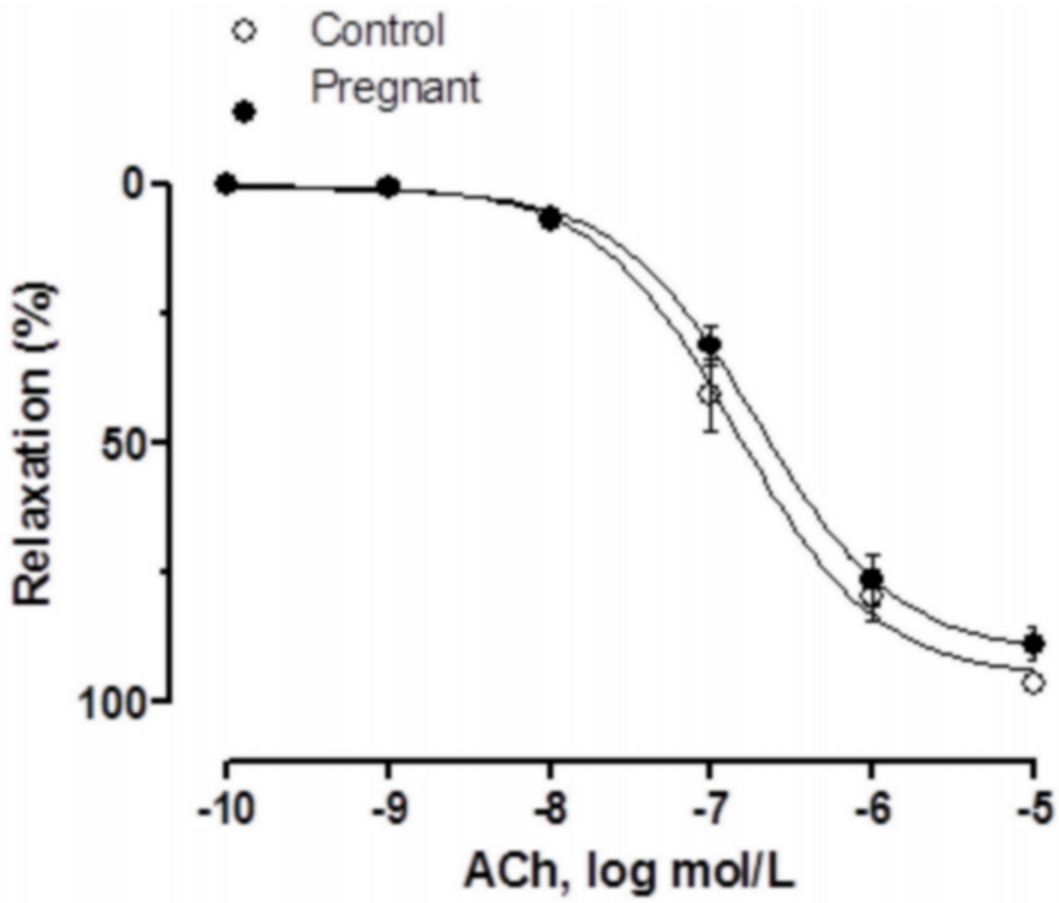

Fig 1. Vasodilator response to ACh. ACh-induced vasodilation in endothelium-intact mesenteric segments from control and pregnant rats. Results (mean ±SEM) were expressed as a percentage of the previous tone elicited by exogenous NA. n = 6 animals each group.

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Effect of pregnancy on the sensory component of vascular responses to EFS in endothelium-denuded mesenteric segments

Preincubation with the CGRP receptor antagonist CGRP (8–37) (0.5 μmol/L) did not alter the EFS-induced contraction in any experimental group, indicating that sensory innervation did not contribute to the observed effects (Fig 4A and 4B).

Table 1. E_{max} (%) and log EC_{50} (mmol/L) values of vasodilator responses to Ach and DEA-NO or vasoconstrictor responses to NA in mesenteric arteries from control and pregnant rats.

	Control		Pregnant	
	E_{max}	-logEC_{50}	E_{max}	-logEC_{50}
ACh	95.09±3.07	6.84±0.08	90.58±2.76	6.72±0.07
NA	167.4±10.2	7.26±0.12	129.7±8.44*	7.23±0.2
DEA-NO	101.8±2.11	6.83±0.06	94.27±3.15	7.21±0.11

Results are expressed as mean±SEM.

*P<0.05 vs. Control.

n = 7 animals each group.

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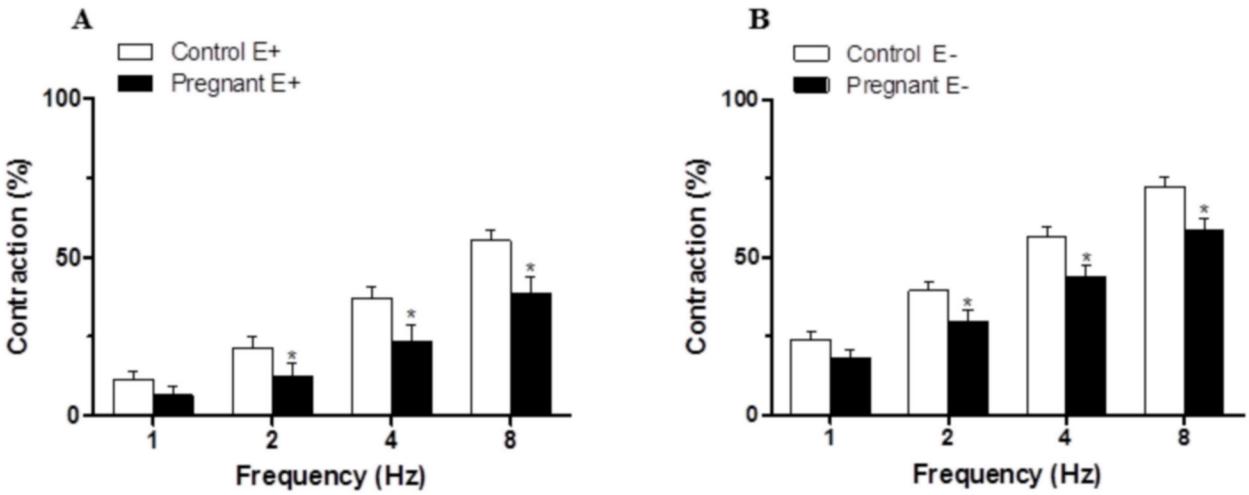

Fig 2. Vasoconstrictor response to EFS. EFS-induced vasoconstriction in endothelium intact (A) and endothelium-denuded (B) mesenteric segments from control and pregnant rats. Results (mean±SEM) were expressed as a percentage of the initial contraction elicited by KCl. ANOVA P<0.05 Control vs. pregnant in both endothelium intact (A) and endothelium-denuded (B). *P<0.05 vs. control animals at each frequency (Bonferroni test). n = 10 animals each group.

doi:10.1371/journal.pone.0126017.g002

Table 2. EFS potentiation after endothelium removal in superior mesenteric artery from control and pregnant rats.

	1 Hz	2 Hz	4 Hz	8 Hz
Control	12.51±2.61	18.19±2.79	19.38±3.05	17.23±3.32
Pregnant	11.55±2.92	17.39±3.85	20.26±4.37	19.79±4.26

Percentage (%) of potentiation in EFS-induced contraction after endothelium removal. Calculations are performed taking KCl-induced contraction as 100% of the contractile response. Results are expressed as mean ± SEM. n = 10 animals each group.

doi:10.1371/journal.pone.0126017.t002

Effect of pregnancy on the nitrergic component of vascular responses to EFS in endothelium-denuded mesenteric segments

Preincubation with the unspecific NOS inhibitor L-NAME (0.1 mmol/L) significantly increased the EFS-contractile response at all frequencies in segments from control and pregnant rats (Fig 5A and 5B). This increase was greater in arteries from pregnant animals (Fig 5C).

Table 3. Effect of preincubation with tetrodotoxin (TTX, 0.1μmol/L) or 6-hydroxydopamine (6-OHDA, 1.46 mmol/L) on the frequency—contraction curves performed in mesenteric segments of control and pregnant rats.

	1 Hz	2 Hz	4 Hz	8 Hz
Control	21.31±2.23	34.10±2.56	52.41±2.22	69.28±3.91
TTX	0	0	0	0.5±0.06
6-OHDA	0	0	0.1±0.02	0.2±0.04
Pregnant	16.42±2.72	25.9±3.58	39.28±4.04	53.75±3.99
TTX	0	0	0	0.4±0.05
6-OHDA	0	0	0.1±0.01	0.3±0.05

Results (means ± S.E.M.) are expressed as percentages of the response elicited by 75 mM KCl; zeros are used when contraction was not detected. n = 7 animals each group.

doi:10.1371/journal.pone.0126017.t003

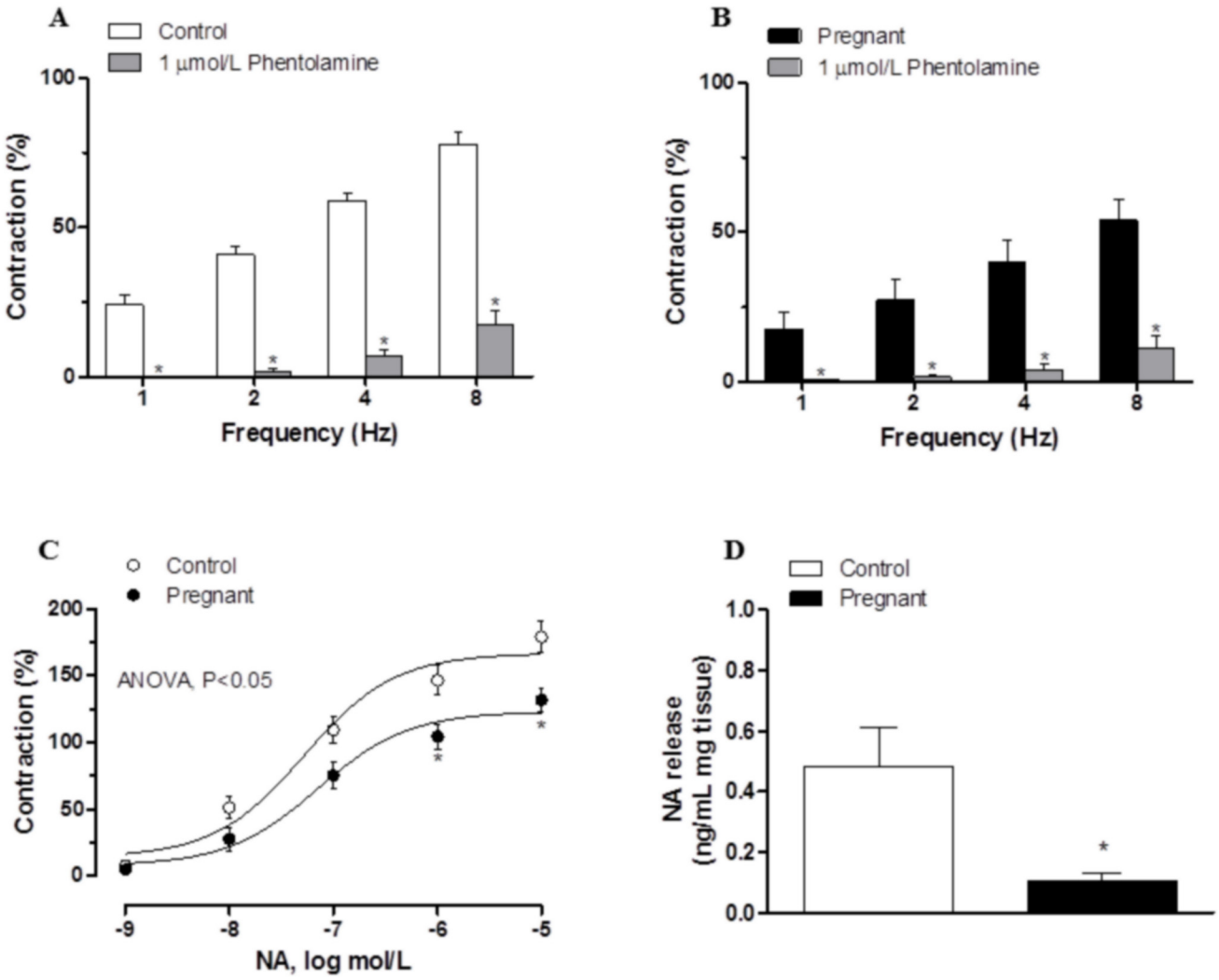

Fig 3. Effect of pregnancy on sympathetic innervation function. Effect of preincubation with 1 µmol/L phentolamine on vasonstriction response induced by EFS in endothelium-denuded mesenteric segments from control (A) and pregnant rats (B). Results (mean±SEM) were expressed as a percentage of the initial contraction elicited by KCl. ANOVA P<0.05 vs. conditions without phentolamine in both experimental groups. *P<0.05 vs. conditions without phentolamine at each frequency (Bonferroni test). n = 8 animals per group. (C) Vasoconstriction response to NA in segments of control and pregnant rats. Results (mean±SEM) were expressed as a percentage of the initial contraction elicited by KCl. ANOVA P<0.05 Control vs. pregnant. *P<0.05 vs. control animals at each concentration (Bonferroni test). n = 8 animals per group. (D) EFS-induced NA release in mesenteric segments of control and pregnant rats. Results expressed as ng NA/mL mg tissue. *P<0.05 vs. Control. n = 6 animals per group.

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EFS-induced NO release was significantly greater in segments from pregnant rats (Fig 6A). Preincubation with 0.1 mmol L-NAME, 0.1 mmol 7-NI or 0.1 µmol/L TTX abolished EFS-induced NO release in both experimental groups (Fig 6A), while preincubation with 1 µmol/L 1400W did not modify this release (Fig 6A). Pregnancy did not modify nNOS expression but did increase P-nNOS expression (Fig 6B).

In segments precontracted with NA, vasodilator response elicited by the exogenous NO donor DEA-NO (0.1 nmol/L- 10 µmol/L) was similar in mesenteric segments from both experimental groups (Fig 7A, Table 2). Preincubation with 0.1 mmol/L tempol augmented DEA-NO-induced vasodilation similarly in segments from control and pregnant rats (Fig 7B and 7C). On the other hand, after subtracting the lucigenin chemiluminescence obtained in the presence of tiron from that obtained in its absence, superoxide anion formation was greater in

Table 4. Effect of preincubation with phentolamine (0.1 μmol/L) on the frequency—contraction curves performed in mesenteric segments from control and pregnant rats.

	1 Hz	2 Hz	4 Hz	8 Hz
Control	24.1±3.23	41.06±2.84	59.14±2.22	77.76±3.90
Phentolamine inhibition	23.79±4.01*	38.88±3.08*	52.90±2.74*	62.63±3.47*
Pregnant	17.53±3.71	27.17±5.84	40.14±5.42	54.03±5.96
Phentolamine inhibition	16.96±5.55*	25.54±6.42*#	36.13±6.39*#	42.97±4.12*#

Percentage (%) of inhibition in EFS-induced contraction after preincubation with phentolamine. Calculations are performed taking KCl-induced contraction as 100% of the contractile response. Results are expressed as mean ± SEM.

*P<0.05 vs. conditions without phentolamine at each frequency (Bonferroni test).

#P<0.05 vs. Control.

n = 8 animals per group.

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Table 5. Remnant vasoconstriction after preincubation with phentolamine (0.1 μmol/L) on the frequency—contraction curves performed in mesenteric segments of control and pregnant rats.

	1 Hz	2 Hz	4 Hz	8 Hz
Control	24.1±3.23	41.06±2.84	59.14±2.22	77.76±3.90
Phentolamine Remnant	0.40±0.19*	2.17±0.88*	7.98±1.38*	18.35±5.54*
Pregnant	17.53±3.71	27.17±5.84	40.14±5.42	54.03±5.96
Phentolamine Remnant	0.61±0.4*	1.63±0.56*	3.72±2.06*#	9.97±3.63*#

Results (means ± S.E.M.) are expressed as percentages of the response elicited by 75 mM KCl.

*P<0.05 vs. conditions without phentolamine at each frequency (Bonferroni test).

#P<0.05 vs. Control.

n = 8 animals per group.

doi:10.1371/journal.pone.0126017.t005

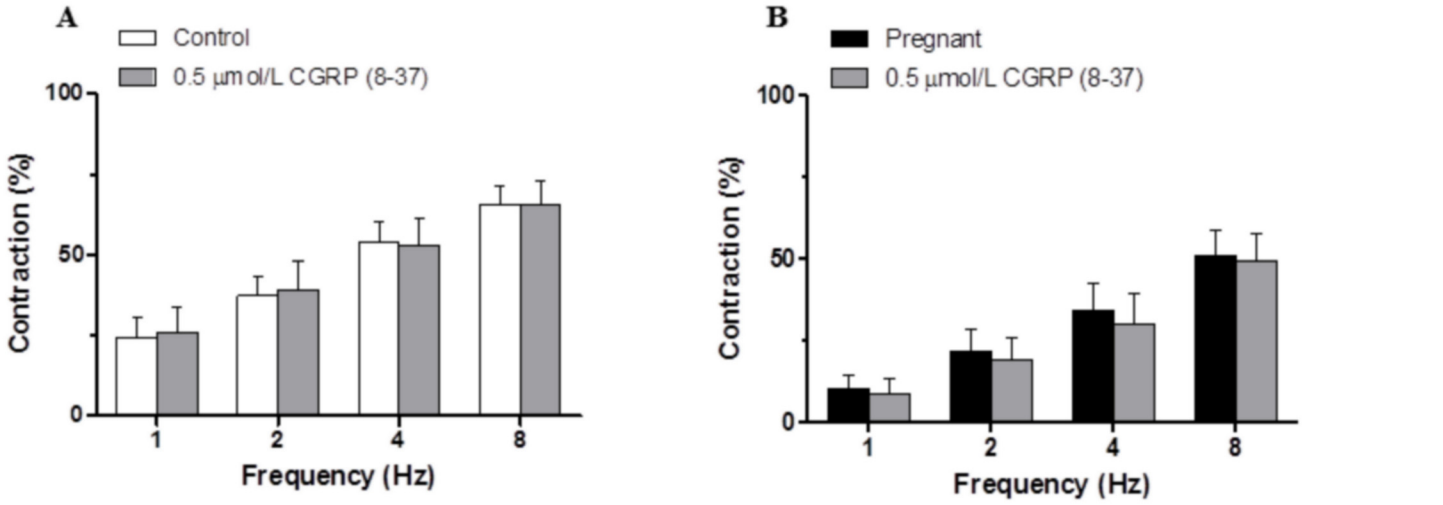

Fig 4. Effect of pregnancy on sensory innervation. Effect of preincubation with 0.5 μmol/L CGRP (8–37) on the vasoconstrictor response induced by EFS in mesenteric segments from (A) control and (B) pregnant rats. Results (mean±SEM) are expressed as a percentage of the previous contraction elicited by KCl. n = 8 animals per group.

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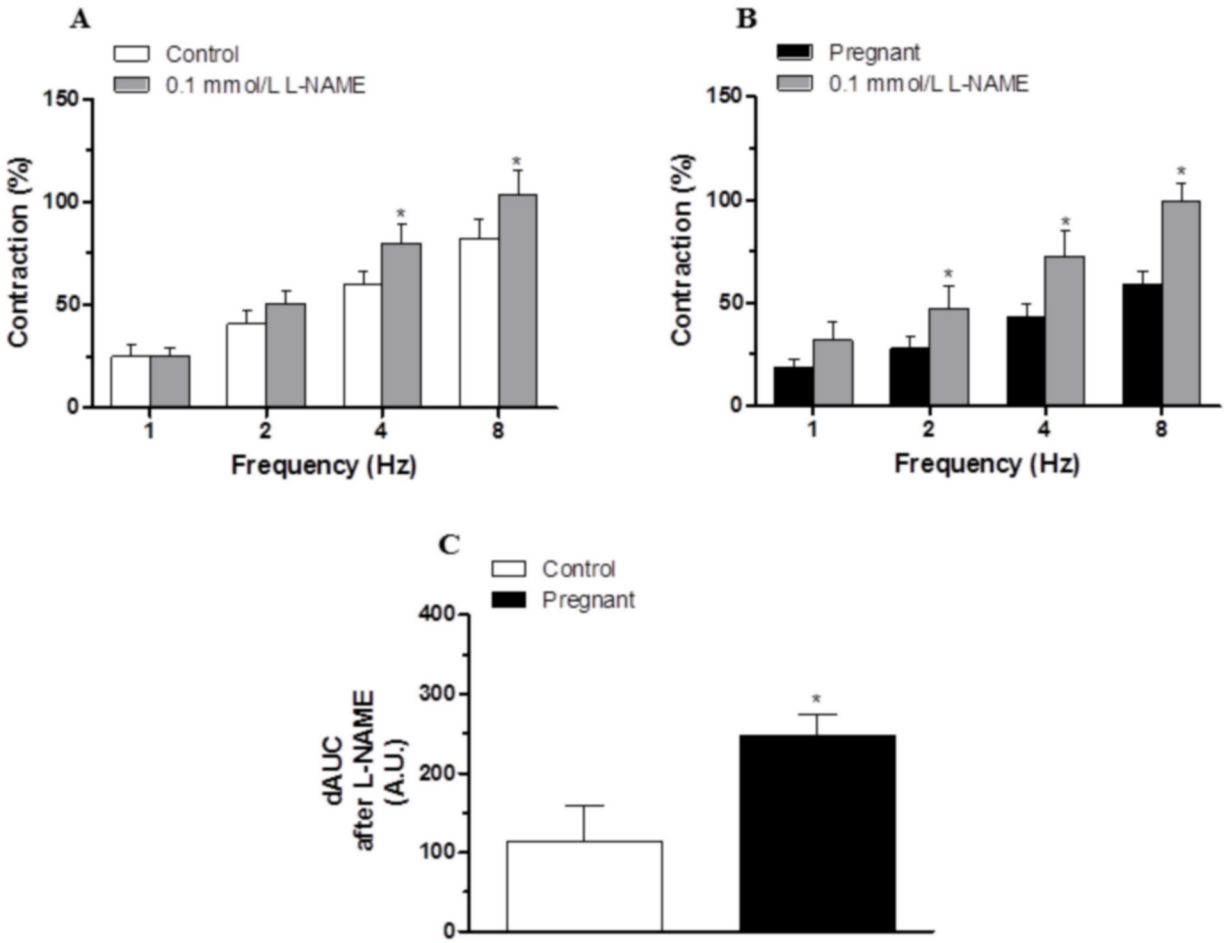

Fig 5. Effect of pregnancy on nitrergic innervation. Effect of preincubation with 0.1 mmol/L L-NAME on the vasoconstrictor response induced by EFS in mesenteric segments from (A) control and (B) pregnant rats. Results (mean±SEM) are expressed as a percentage of the previous contraction elicited by KCl. ANOVA P<0.05 vs. conditions without L-NAME in both experimental groups. *P<0.05 vs. conditions without L-NAME for each frequency (Bonferroni test). n = 8 animals per group. (C) Differences of area under curve (dAUC) in the absence or presence of 0.1 mmol/L L-NAME, dAUC values are expressed as arbitrary units.*P<0.05 Control vs. pregnant rats.

doi:10.1371/journal.pone.0126017.g005

arteries from pregnant rats (Fig 7D). Preincubation with apocinin decreased superoxide anion formation significantly in mesenteric segments of pregnant rats, while allopurinol did not modify this formation in any experimental group (Fig 7D)

Discussion

The present study shows that pregnancy diminished the vasoconstrictor response due to stimulation of perivascular innervation in mesenteric artery through an endothelium-independent mechanism. This effect is associated with a reduction in sympathetic innervation function mediated by a decreased NA release and vasoconstrictor response and an increased nitrergic function associated with increased neuronal NO release. Sensory innervation did not participate in any experimental conditions.

When analyzing the vasoconstrictor response induced by EFS in endothelium-intact segments, we observed that EFS produced a frequency-dependent contraction in segments from both control and pregnant rats that was lower in segments from pregnant rats, as previously reported [4,25,26]. The fact that in our experimental conditions the vasoconstrictor response to

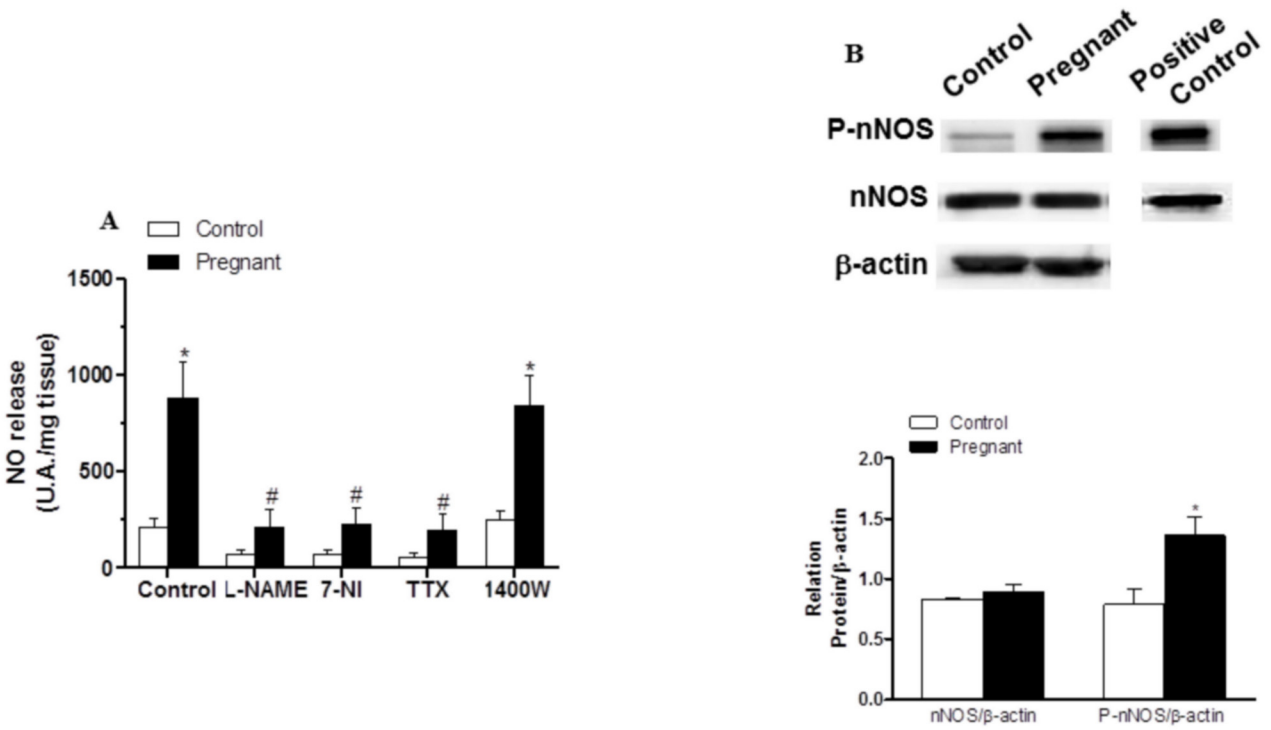

Fig 6. Effect of pregnancy on neuronal NO synthesis. (A) Effect of 0.1 mmo/L L-NAME or 0.1 mmol/L 7-NI or 0.1 μmol/L TTX on EFS-induced NO release in segments from control and pregnant rats. Results (mean±SEM) were expressed as arbitrary (A.U.)/mg tissue. *P<0.05 vs. Control; #P<0.05 compared with conditions without specific inhibitor; n = 8 animals per group. (B) Effect of pregnancy on nNOS and P-nNOS expression. The blot is representative of four separate segments from each group. Rat brain homogenates were used as a positive control. Lower panel shows relation between P-nNOS or nNOS expression and β-actin. Results (mean±SEM) are expressed as ratio of the signal obtained for each protein and the signal obtained for β-actin. *P<0.05 Control vs. pregnant rats.

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KCl remained unmodified by pregnancy, as has been previously reported in this artery [4,27], indicates the maintenance of vasoconstrictor capacity, ruling out possible changes in the intrinsic contractile machinery of smooth muscle cells.

It has been widely described that endothelium affects the response to several vasomotor substances, including neurotransmitters [28], and since pregnancy could affect the production of endothelial factors [29] it would consequently affect these responses. In our experimental conditions ACh-induced vasodilation was not modified by pregnancy as also reported by Ralevic and Burnstock at late-term pregnancy [4]. Endothelium removal increased vasoconstrictor response to EFS similarly in both groups, indicating that the modulating role of endothelium in the EFS vasoconstrictor response is unaltered by pregnancy so the decreased contractile response would be due to modifications in perivascular innervation function produced by an endothelium-independent mechanism. Taking into account these results, we performed the following experiments only in endothelium-denuded mesenteric segments. The abolishment of EFS-induced vasoconstriction in the presence of TTX indicates that the response to EFS is due to neurotransmitter release. Sympathetic and nitrergic innervation involvement in the vasomotor response to EFS in rat superior mesenteric artery has been widely reported [9,10], while sensory innervation participation only appears in several pathological situations [13,30,31,32,33].

A decreased participation of sympathetic innervation in splanchnic blood flow as an adaptation to pregnancy has been demonstrated previously and associated with decreased vasoconstrictor response to NA [7]. However the possibility that the NA release was also affected by

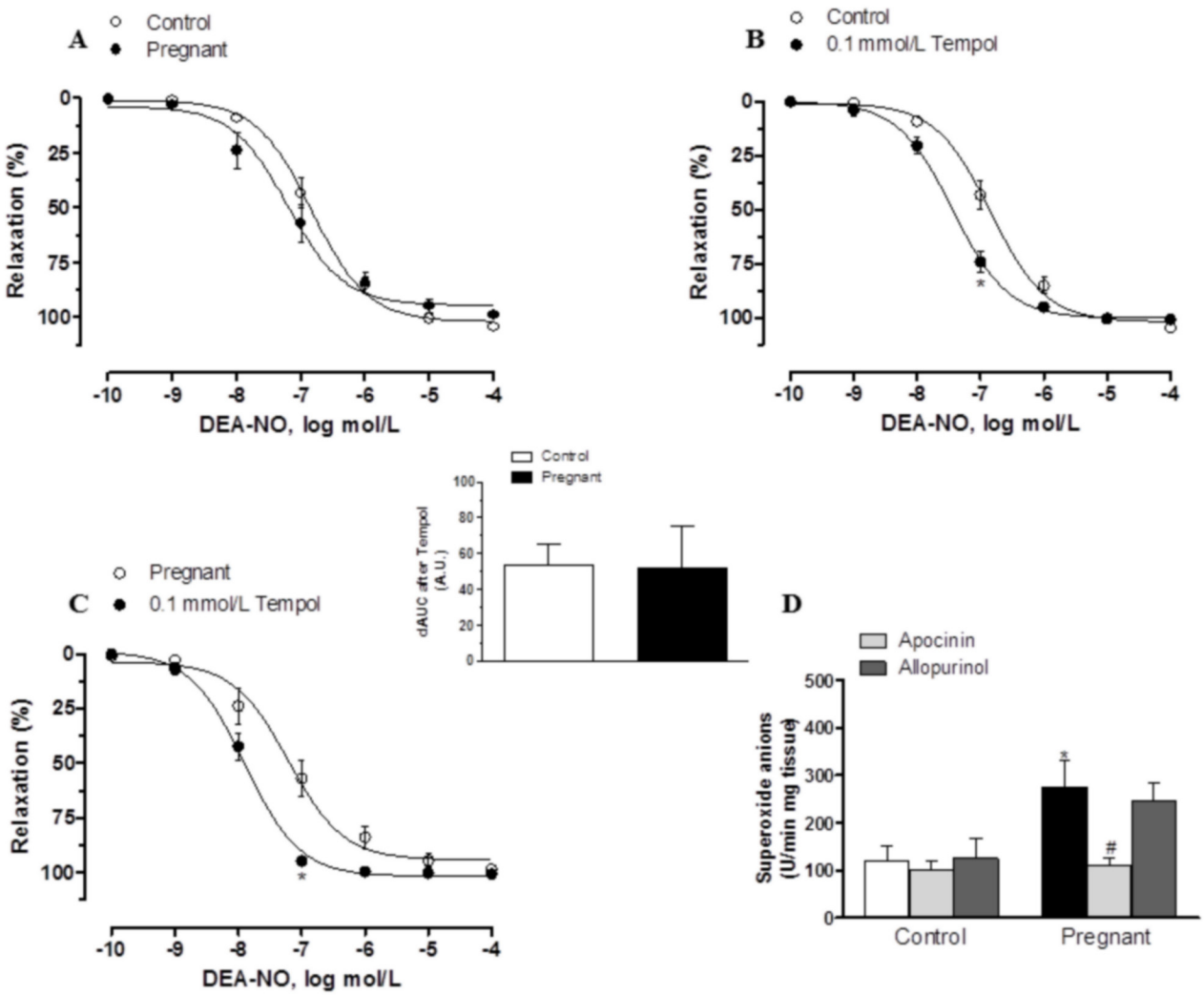

Fig 7. Effect of pregnancy on DEA-NO vasodilation and superoxide anion production. (A) Vasodilator response to NO donor DEA-NO in segments from control and pregnant rats. Effect of preincubation with tempol (0.1 mmol/L) on vasodilator response to NO donor DEA-NO in segments from (B) control and (C) pregnant rats. Results (mean±SEM) are expressed as a percentage of the previous tone elicited by exogenous NA. ANOVA P<0.05 vs. conditions without tempol in both experimental groups. *P<0.05 vs. conditions without tempol for each concentration (Bonferroni test). n = 7 animals per group. Insert graph shows differences of area under curve (dAUC) in the absence or presence of 0.1 mmol/L tempol; dAUC values are expressed as arbitrary units. (D) Effect of 0.3 mmol/L apocinin or 0.1 mmol/L allopurinol on superoxide anion release in mesenteric segments from control and pregnant rats. Results (mean ±SEM) are expressed as chemoluminiscence units (U)/min mg tissue. *P<0.05 vs. Control; #P<0.05 compared with conditions without specific inhibitor; n = 7 animals per group.

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pregnancy has been also suggested but not measured [3,4]. Preincubation with the alpha-adrenoceptor antagonist phentolamine significantly diminished the vasoconstrictor response to EFS in mesenteric segments from both experimental groups. This decrease was less marked in mesenteric segments from pregnant rats, indicating a decreased participation by the adrenergic component from sympathetic innervation in the lower vasoconstrictor response to EFS in pregnant rats, similarly to results described by Ralevic and Burnstock [4]. The differences in the vasoconstrictor response to EFS could be associated with changes in neurotransmitter release and/or vasomotor response to NA. The results obtained demonstrate that late pregnancy decreases the vasoconstrictor response to NA and simultaneously decreases NA release in mesenteric artery. It is well known that there is a decrease in vasomotor responses to contractile

substances in pregnancy, although the mechanisms implicated are not well known. No changes in alpha-adrenoceptor affinity in pregnancy [34], decreased alpha(1)-adrenoceptor expression [35] and modifications in the regulation of Ca(2+) mobilization and Ca(2+) sensitivity in alpha (1)-adrenoceptor-mediated contractions [36] have all been described. While the decrease in NA release could be associated with changes in sympathetic nerve density due to estrogens, previous studies have report that pregnancy produces no change [37], increased [38] and decreased [39] sympathetic nerve density, depending on the tissue studied. We have reported that the remnant vasoconstriction after preincubation with phentolamine is due to the release of sympathetic cotransmitter ATP in mesenteric segments from female rats [14]. Preincubation with the neurotoxine 6-OHDA practically abolished EFS-induced contraction in both experimental groups while the remnant vasoconstriction after preincubation with phentolamine was decreased in mesenteric segments of pregnant rats, suggesting a minor role for ATP in late pregnancy. The diminished sympathetic innervation function could, by itself, explain the decreased EFS-induced vasoconstriction observed in mesenteric segments from pregnant rats. However, the participation of other neural components cannot be ruled out.

We have previously reported that sensory innervation does not participate in vasomotor response induced by EFS in control Sprague Dawley rats [14]. However, the participation of this innervation is increased in several pathological conditions such as hypertension, cirrhosis and diabetes [20,30,31,32]. Important variations in serum levels of the vasodilator CGRP have been reported in humans and rats during pregnancy [40,41]. Additionally, in rat mesenteric arteries, both early and late pregnancy are associated with a rise in the vascular sensitivity to CGRP in selected areas of the vascular bed without a concomitant increase in vascular CGRP production and release [15,16,42]. This observation led us to study the possible role of sensory innervation in the decreased EFS vasoconstriction observed in pregnant rats. Preincubation with the CGRP receptor antagonist CGRP (8–37) did not alter EFS-induced vasoconstriction in any experimental group, indicating that in late pregnancy sensory innervation does not participate in the vasoconstrictor response to EFS. This result contrasts with those of Ralevic and Burnstock [4], who observed a participation of sensory innervation in the EFS-mediated relaxation, and this response was not modified by pregnancy. Nevertheless, Lanlua *et al.* [43] reported no effect from CGRP (8–37) in the EFS-mediated relaxation of small mesenteric arteries in pregnancy.

It has also been suggested an involvement by NO in the decreased vasoconstrictor response to EFS in conductance and resistance mesenteric arteries during pregnancy [4,25] since there is increased NO production and release by endothelial cells. However, the fact that the unspecific inhibition of NO synthesis also affects neuronal NO does not rule out the participation of nitrergic perivascular innervation, especially when NO released from nitrergic innervation has been demonstrated to have a vasodilator role that contributes to decreasing the vasomotor response to EFS [10]. Previously, we have demonstrated that both the non-selective NOS inhibitor L-NAME and the specific nNOS inhibitor 7-NI decrease EFS-induced NO release to a similar extent [23]. However, in vascular reactivity experiments, preincubation with 7-NI also decreased the vasoconstrictor response to NA, making the analysis of EFS-induced contractions very complex, and leading to result misinterpretation [23,44]. For that reason, we used L-NAME instead of 7-NI in vascular reactivity experiments. Vasoconstriction induced by EFS was increased in L-NAME incubated segments from both groups of rats, suggesting an involvement by NO in this response, as previously described in this rat strain [10]. The greater effect of L-NAME observed in segments from pregnant rats suggests an increased role of neuronal NO in EFS-induced vasoconstriction related to increases in NO release and/or increases in the vasodilator response. EFS-induced NO release was higher in segments from pregnant animals, confirming our hypothesis. Production of NO in neural tissue can have two sources: nNOS and iNOS [22,44,45]. EFS-induced NO release was abolished by preincubation with L-NAME, 7-NI or

TTX in both experimental groups, confirming that EFS induced NO release from nitrergic innervation. Additionally, in the present study, the specific iNOS inhibitor 1400W did not change EFS-induced NO release in segments from the two experimental groups, ruling out the participation of this inducible isoform in our experimental approach, as previously reported [22,46].

The differences in NO release observed in our experimental conditions could be dependent on nNOS expression and/or activity. We found that nNOS protein expression was not modified by pregnancy, whereas P-nNOS, the active form of nNOS was increased, suggesting increased nNOS activity. Estrogens are reported to regulate constitutive nitric oxide synthases and this regulation could be implicated in changes occurring in pregnancy [47]. Additionally Martini *et al.* [48] have reported an increase in nNOS expression correlated with increased female sex hormone levels, suggesting a complex action by female sex hormones in nNOS expression; therefore the increased nNOS activity could be due to the estrogens as has been reported previously [49].

Thus, increased NO release participates in the diminished response to EFS in segments from pregnant rats. However, altered smooth muscle cell sensitivity to NO cannot be ruled out since measurements of oxidative stress markers in maternal blood and urine show that pregnancy itself is a state of oxidative stress due to the high metabolic activity of the placenta and maternal metabolism [50], and this could alter NO bioavailability. First, we confirmed that superoxide anion production is increased in mesenteric segments from pregnant rats. Oxidative stress in pregnancy is regulated differently depending on the organ analysed [51]. It is well known that NADPH oxidase is the main source of superoxide anion in vessels [52]. Taking this into account, we have measured superoxide anion production in the presence of the NADPH oxidase inhibitor apocinin and the xanthine oxidase inhibitor allopurinol. Preincubation with apocinin significantly decreased superoxide anion production only in mesenteric segments of pregnant rats, but allopurinol did not modify superoxide anion production in any experimental group. These results confirm NADPH oxidase as the principal source of oxidative stress in superior mesenteric artery in late pregnancy. However, the vasodilator response to DEA-NO was similar in segments from both experimental groups. Additionally, preincubation with the superoxide anion scavenger tempol increased vasodilator response to DEA-NO to a similar extent in mesenteric segments from both control and pregnant rats. These results suggest that, despite the increased superoxide anion levels observed, the oxidation process is unable to affect the NO metabolism enough to significantly affect vasodilator response.

In conclusion, our results show that neural control of mesenteric vasomotor tone was altered by pregnancy. Diminished sympathetic and enhanced nitrergic components both contributed to a decreased vasoconstriction response to EFS. The sensory innervation did not participate in these modifications. All these changes indicate the simultaneous implication of different components of perivascular innervation in vascular adaptations to pregnancy.

Author Contributions

Conceived and designed the experiments: ES JBR LC GB. Performed the experiments: ES JBR LC MC. Analyzed the data: ES JBR LC MC GB. Contributed reagents/materials/analysis tools: JBR GB. Wrote the paper: ES JBR LC GB.

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Artículo 2: Breast feeding increases vasoconstriction induced by electrical field stimulation in rat mesenteric artery. Role of neuronal nitric oxide and ATP.

Sastre E, Blanco-Rivero J, Sastre E, Caracuel L, Granado M, Balfagón G

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Breast Feeding Increases Vasoconstriction Induced by Electrical Field Stimulation in Rat Mesenteric Artery. Role of Neuronal Nitric Oxide and ATP

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Abstract

Objectives: The aim of this study was to investigate in rat mesenteric artery whether breast feeding (BF) affects the vasomotor response induced by electrical field stimulation (EFS), participation by different innervations in the EFS-induced response and the mechanism/s underlying these possible modifications.

Methods: Experiments were performed in female Sprague-Dawley rats (3 months old), divided into three groups: Control (in oestrous phase), mothers after 21 days of BF, and mothers that had recovered their oestral cycle (After BF, in oestrous phase). Vasomotor response to EFS, noradrenaline (NA) and nitric oxide (NO) donor DEA-NO were studied. Neuronal NO synthase (nNOS) and phosphorylated nNOS (P-nNOS) protein expression were analysed and NO, superoxide anion ($O_2^{\cdot-}$), NA and ATP releases were also determined.

Results: EFS-induced contraction was higher in the BF group, and was recovered after BF. 1 μmol/L phentolamine decreased the response to EFS similarly in control and BF rats. NA vasoconstriction and release were similar in both experimental groups. ATP release was higher in segments from BF rats. 0.1 mmol/L L-NAME increased the response to EFS in both control and BF rats, but more so in control animals. BF decreased NO release and did not modify $O_2^{\cdot-}$ production. Vasodilator response to DEA-NO was similar in both groups, while nNOS and P-nNOS expressions were decreased in segments from BF animals.

Conclusion: Breast feeding increases EFS-induced contraction in mesenteric arteries, mainly through the decrease of neuronal NO release mediated by decreased nNOS and P-nNOS expression. Sympathetic function is increased through the increased ATP release in BF rats.

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Introduction

Breast feeding is characterised by strict hormonal control mediated by oestrogen, progesterone, prolactin and oxytocin, associated with increases in gastrointestinal blood flow and cardiac output [1,2] to provide the udder with nutrients and hormones that regulate milk synthesis and secretion. Some hormones implicated in the lactation process (prolactin, oxytocin) have been reported to alter vascular function [3,4].

Vascular tone is determined by an equilibrium among several mechanisms, including hormonal, metabolic and neuronal factors. This neural regulation involves sympathetic, cholinergic, nitrergic, peptidergic and/or sensory innervations that are specific to the vascular bed considered [5,6]. In rat mesenteric arteries, vascular tone is mediated by the integrated action of different neurotransmitters, mainly noradrenaline (NA) and ATP from sympathetic nerve terminals, neuronal nitric oxide (NO) from nitrergic innervation and calcitonin gene-related peptide (CGRP) from sensory nerves [7,8,9,10]. Several experimental and pathophysiological circumstances including ageing, hypertension and diabetes, have been shown to alter the functional role of these components [11,12,13].

We have previously reported that sex steroids modulate the synthesis and/or sensitivity to these neurotransmitters in rat mesenteric arteries [14,15]. Reports of the effects of breast feeding on sympathetic activity are contradictory, with increased and decreased sympathetic tone being described [16,17,18]. On the other hand, increased serum nitrite and nitrate concentrations [19] and ROS generation [20] have been reported in breast-feeding mothers. These findings indicate that the functional role of adrenergic and nitrergic innervations could be affected in breast feeding and this would affect peripheral vascular resistance.

Therefore, the aim of this study was to investigate whether breast feeding affects neurotransmitter release or EFS-induced vasomotor response in rat mesenteric arteries, the participation of

different innervations in the EFS-induced response and the mechanism/s underlying these possible modifications.

Methods

Animals

Female Sprague-Dawley rats (3 months-old) were obtained from the Animal Quarters of the Universidad Autónoma de Madrid and housed in the Animal Facility of the Universidad Autónoma de Madrid (Registration number EX-021U) in accordance with guidelines 609/86 of the E.E.C., R.D. 233/88 of the *Ministerio de Agricultura, Pesca y Alimentación* of Spain, and Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health [NIH publication No. 85.23, revised 1985]. All experimental procedures involving animal use were approved by the Ethics Committee of the *Universidad Autónoma de Madrid*. Rats were housed during treatment at a constant room temperature, humidity, and light cycle (12:12 h light-dark) with free access to tap water and fed with standard rat chow *ad libitum*. Rats were divided into three groups: Control (virgin females in oestrous phase), mothers after 21 days of breast feeding (BF), and mothers that had recovered their oestral cycle (After BF, in oestrous phase).

Animals were sacrificed by CO_2 inhalation; the first branch of the mesenteric artery was carefully dissected, cleaned of connective tissue and placed in Krebs-Henseleit solution (KHS, in mmol/L: NaCl 115, $CaCl_2$ 2.5, KCl 4.6, KH_2PO_4 1.2, $MgSO_4$. $7H_2O$ 1.2, $NaHCO_3$ 25, glucose 11.1, Na_2EDTA 0.03) at 4°C. For protein expression analysis, some arteries were rapidly frozen in liquid nitrogen and kept at −80°C until the day of analysis.

Vascular Reactivity

The method used for isometric tension recording has been described in full elsewhere [9,21]. Two parallel stainless steel pins were introduced through the lumen of the vascular segment: one was fixed to the bath wall and the other connected to a force transducer (Grass FTO3C; Quincy, Mass., USA); this, in turn, was connected to a model 7D Grass polygraph. For EFS experiments, segments were mounted between two platinum electrodes 0.5 cm apart and connected to a stimulator (Grass, model S44) modified to supply adequate current strength. Segments were suspended in an organ bath containing 5 mL of KHS at 37°C and continuously bubbled with a 95% O_2 to 5% CO_2 mixture (pH 7.4). Some experiments were performed in endothelium-denuded segments to eliminate the main source of vasoactive substances, including endothelial NO. This avoided possible actions by different drugs on endothelial cells that could lead to misinterpretation of results. Endothelium was removed by gently rubbing the luminal surface of the segments with a thin wooden stick. The segments were subjected to a tension of 0.5 g, which was readjusted every 15 min during a 90-min equilibration period before drug administration. After this, the vessels were exposed to 75 mmol/L KCl, to check their functional integrity. Endothelium removal did not alter the contractions elicited by 75 mmol/L KCl. After a washout period, the presence/absence of vascular endothelium was tested by the ability/inability of 10 μmol/L acetylcholine (ACh) to relax segments precontracted with NA (1 μmol/L).

Vasodilator response to ACh (0.1 nmol/L-10 μmol/L) was tested in endothelium-intact arteries from all experimental groups.

Frequency-response curves to EFS (1, 2, 4, 8 and 16 Hz) were performed in endothelium-intact and endothelium-removed mesenteric segments from all experimental groups. The parameters used for EFS were 200 mA, 0.3 ms, 1–16 Hz, for 30 s with an interval of 1 min between each stimulus, the time required to recover basal tone. A washout period of at least 1 h was necessary to avoid desensitisation between consecutive curves. Two successive frequency-response curves separated by 1-hour intervals produced similar contractile responses. To evaluate the neural origin of the EFS-induced contractile response, the nerve impulse propagation blocker, tetrodotoxin, (TTX, 0.1 μmol/L) was added to the bath 30 min before the second frequency-response curve was performed.

To determine the participation of sympathetic innervation in the EFS-induced response in endothelium-denuded segments from control and BF rats, 1 μmol/L phentolamine, an α-adrenoceptor antagonist, or phentolamine plus 0.1 mmol/L suramin, a non-specific P2 purinergic receptor antagonist, were added to the bath 30 min before performing the frequency-response curve. Additionally, the vasoconstrictor response to exogenous NA (1 nmol/L-10 μmol/L) was tested in segments from both experimental groups.

To study the possible participation of sensitive innervation in EFS-induced response in endothelium-denuded segments from control and BF rats, 0.5 μmol/L CGRP (8–37), a CGRP receptor antagonist, was added to the bath 30 min before performing the second frequency–response curve.

To analyse the participation of NO in the EFS- induced response in endothelium-denuded segments from control and BF rats, 0.1 mmol/L N^{ω}-nitro-L-arginine methyl ester (L-NAME), a non-specific inhibitor of nitric oxide synthase (NOS), was added to the bath 30 min before performing the second frequency–response curve. The vasodilator response to the NO donor, diethylamine NONOate, (DEA-NO, 0.1 nmol/L–0.1 mmol/L) was determined in NA-precontracted arteries from both experimental groups.

Noradrenaline and ATP Release

Endothelium-denuded segments of rat mesenteric arteries from control and BF rats were preincubated for 30 minutes in 5 mL of KHS at 37°C and continuously gassed with a 95% O_2–5% CO_2 mixture (stabilisation period). This was followed by two washout periods of 10 min in a bath of 0.4 mL KHS. Then the medium was collected to measure basal release. Next, the organ bath was refilled and cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz were applied at 1 min intervals. Afterwards, the medium was collected to measure EFS-induced neurotransmitter release. Mesenteric segments were weighed in order to normalise the results.

NA and ATP releases were measured using Noradrenaline Research EIA (Labor Diagnostica Nord, Gmbh and Co., KG, Nordhon, Germany) or an ATP Colorimetric/Fluorometric Assay kit (Abcam, Cambridge, UK). The assays were performed following the manufacturers' instructions. Results were expressed as ng NA/mL mg tissue, or nmol ATP/mL mg tissue.

Nitric Oxide Release

NO release was measured using fluorescence emitted by the fluorescent probe 4,5-diaminofluorescein (DAF-2). Endothelium-denuded mesenteric arteries from control and BF rats were subjected to a 60-minute equilibration period in HEPES buffer (in mmol/L: NaCl 119; HEPES 20; $CaCl_2$ 1.2; KCl 4.6; $MgSO_4$ 1; KH_2PO_4 0.4; $NaHCO_3$ 5; glucose 5.5; Na_2HPO_4 0.15; pH 7.4) at 37°C. Arteries were incubated with 2 μmol/L DAF-2 for 30 min. The medium was then collected to measure basal NO release. Once the organ bath was refilled, cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz were applied at 1 min intervals. Afterwards, the medium was collected to measure EFS-induced NO release. The fluorescence of the medium was measured at room temperature using a spectrofluorometer (LS50 Perkin Elmer

Instruments, FL WINLAB Software, Whaltmann, MA, USA) with excitation wavelength set at 492 nm and emission wavelength at 515 nm.

The EFS-induced NO release was calculated by subtracting basal NO release from that evoked by EFS. Also, blank sample measures were collected in the same way from segment-free medium in order to subtract background emission. Some assays were performed in the presence of 0.1 μmol//L TTX or 1 μmol/L 1400W, the specific iNOS inhibitor. The amount of NO released was expressed as arbitrary units/mg tissue.

Detection of $O_2^{\cdot-}$

$O_2^{\cdot-}$ levels were measured using lucigenin chemiluminescence. Endothelium-denuded mesenteric segments from control and BF rats were rinsed in KHS for 30 min, equilibrated for 30 min in HEPES buffer at 37°C, transferred to test tubes that contained 1 mL HEPES buffer (pH 7.4) containing lucigenin (5 μmol/L) and then kept at 37°C. The luminometer was set to report arbitrary units of emitted light; repeated measurements were collected for 5 min at 10 s intervals and averaged. 4,5-Dihydroxy-1,3-benzene-disulphonic acid "Tiron" (10 mmol/L), a cell-permeant, non-enzymatic $O_2^{\cdot-}$ scavenger, was added to quench the $O_2^{\cdot-}$dependent chemiluminescence. Also, blank samples were collected in the same way without mesenteric segments to subtract background emission.

nNOs and P-nNOS Expression

Western blot analysis of nNOS and phosphorylated nNOS (P-nNOS) expression was performed in endothelium-denuded mesenteric segments from control and BF rats, as previously described [22]. For these experiments, we used mouse monoclonal nNOS antibody (1:1000, Transduction Laboratories), rabbit polyclonal P-nNOS antibody (1:2000, Abcam, Cambridge, UK), and monoclonal anti-ß -actin-peroxidase antibody (1:50000, Sigma-Aldrich, Spain). Rat brain homogenates were used as positive control.

Drugs Used

L -NA hydrochloride, ACh chloride, diethylamine NONOate diethylammonium salt, CGRP (8–37), suramin hexasodium salt, TTX, L -NAME hydrochloride, 1400W, phentolamine, lucigenin, tiron and DAF-2 (Sigma-Aldrich, Madrid, Spain) were used. Stock solutions (10 mmol/L) of drugs were made in distilled water, except for NA, which was dissolved in a NaCl (0.9%)-ascorbic acid (0.01% w/v) solution. These solutions were kept at –20°C and appropriate dilutions were made in KHS on the day of the experiment.

Data Analysis

The responses elicited by EFS and NA were expressed as a percentage of the initial contraction elicited by 75 mmol/L KCl for comparison between control and BF rats. The relaxation induced by ACh or DEA-NO was expressed as a percentage of the initial contraction elicited by NA (Control: 1070.54±4.6 mg; BF: 1096.92±5.1 mg; after BF: 1054.17±8.5; $P>0.05$). For concentration-response curves, non-linear regression and E_{max} and –log EC_{50} were performed. Results are given as mean±SEM. Statistical analysis was done by comparing the curve obtained in the presence of the different substances with the previous or control curve by means of repeated measure analysis of variance (ANOVA) followed by Bonferroni post-hoc test, using GraphPad Prism 5.0 software (CA, USA). Some results were expressed as differences of area under the curve (dAUC) of EFS obtained in segments from control and BF animals. AUC were calculated from the individual concentration-response plots. For dAUC, NO, $O_2^{\cdot-}$, ATP and NA release data, the statistical analysis was done using one-way ANOVA followed by Newman-Keuls post-hoc test. $P<0.05$ was considered significant.

Results

Vasomotor Response to KCl

In endothelium-intact mesenteric segments, the vasoconstrictor response to 75 mmol/l KCl was similar in all experimental groups (Control: 1393±89.65 mg; BF: 1445±164.85 mg; After BF: 1412±120.54 mg; $P>0.05$). Endothelium removal did not alter KCl-induced vasoconstriction (Control: 1348±97.95 mg; BF: 1354±85.77 mg; After BF: 1395±124.46 mg; $P>0.05$).

Vasodilator Response to ACh

Vasodilator response to ACh was similar in all experimental groups (Figure 1, Table 1).

Vascular Responses to EFS

The application of EFS induced a frequency-dependent contractile response in endothelium-intact mesenteric segments from all experimental groups. This vasoconstriction was greater in segments from BF rats compared to control and after BF animals (Figure 2A). Endothelium removal increased EFS-induced contractile response similarly in segments from all experimental groups (Figure 2B, Table 2). EFS-induced contractions were practically abolished in segments from all experimental groups by the neurotoxin TTX (0.1 μmol/L), indicating the neuronal origin of the factors inducing this response (Results not shown).

Since the differences appear only between control and BF rats, and both endothelial and neuronal function are reversed to control situation in after BF rats, we performed the following experiments only in mesenteric segments from control and BF animals.

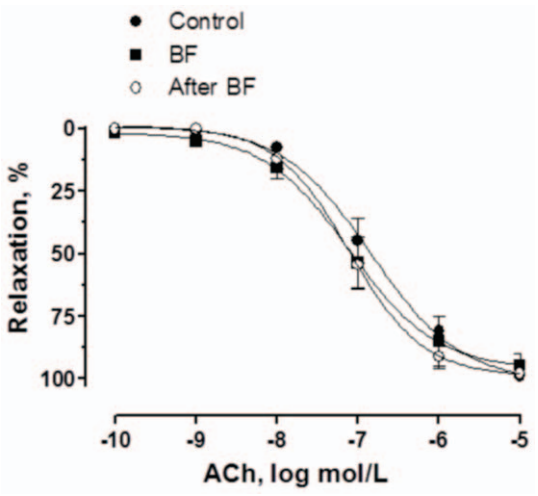

Figure 1. Vasodilator response to ACh. ACh-induced vasodilation in endothelium-intact mesenteric segments from control, breast-feeding (BF) and after BF rats. Results (mean±SEM) were expressed as a percentage of the previous tone elicited by exogenous NA. n = 6 animals each group.
doi:10.1371/journal.pone.0053802.g001

Table 1. E_{max} (%) and log EC_{50} (mmol/L) values of vasodilator responses to ACh in mesenteric arteries from control, BF and after BF female rats.

	Control	BF	After BF
E_{max}	101.5±6.7	96.20±7.4	98.92±4.5
$-\log EC_{50}$	6.94±0.2	7.10±0.3	7.10±0.1

Results are expressed as means±S.E.M. n = 6 animals each group.
doi:10.1371/journal.pone.0053802.t001

Effect of BF on Sympathetic Component of Vascular Responses to EFS

Preincubation with the α-adrenergic antagonist phentolamine (1 μmol/L) decreased the vasoconstrictor response induced by EFS in segments from both experimental groups to the same extent (Figure 3A, 3B and 3C). In agreement with these results, the contraction elicited by exogenous NA (1 nmol/L-10 μmol/L) was similar in segments from both experimental groups (Figure 4A, Table 3). Additionally, EFS-induced NA release was similar in segments from both experimental groups (Figure 4B).

Nevertheless, the remnant vasoconstriction induced by EFS after phentolamine preincubation was greater in segments from BF animals. Preincubation with phentolamine plus 0.1 mmol/L suramin, a non-specific P2 purinergic receptor antagonist, decreased EFS-induced contraction in segments from both experimental groups. This decrease was greater in segments from BF animals (Figure 3A, 3B and 3D). In line with this, EFS-induced ATP release was increased in segments from BF rats (Figure 4C).

Effect of BF on Sensory Component of Vascular Responses to EFS

Preincubation with the CGRP receptor antagonist CGRP (8–37) (0.5 μmol/L) did not alter the EFS-induced contraction in any experimental group (Results not shown).

Table 2. EFS potentiation after endothelium removal.

	1 Hz	2 Hz	4 Hz	8 Hz	16 Hz
Control	16.9±3.5	19.2±2.1	22.4±2.3	18.1±2.5	15.7±2.9
BF	21.2±3.1	22.4±1.8	24.3±3.4	21.7±2.7	18.3±1.7
After BF	17.6±2.9	20.7±1.7	21.9±2.9	19.2±3.1	17.6±2.1

Percentage (%) of potentiation in EFS-induced contraction after endothelium removal. Calculations are performed taking KCl-induced contraction as 100% of the contractile response. Results are expressed as mean ± SEM. n = 10 animals each group.
doi:10.1371/journal.pone.0053802.t002

Effect of BF on Nitrergic Component of Vascular Responses to EFS

EFS-induced NO release was significantly lower in segments from BF rats (Figure 5A). Preincubation with 0.1 μmol/L TTX abolished EFS-induced NO release, while preincubation with 1 μmol/L of the specific iNOS inhibitor 1400W did not modify it (Results not shown). Both nNOS and P-nNOS expression were diminished in segments from BF rats (Figure 5B). In segments precontracted with NA, vasodilator response elicited by the exogenous NO donor DEA-NO (0.1 nmol/L-10 μmol/L) was similar in mesenteric segments from both experimental groups (Figure 5C, Table 3). Additionally, after subtracting the lucigenin chemiluminescence obtained in the presence of Tiron from that obtained in its absence, superoxide anion formation was similar in segments from both experimental groups (Figure 5D).

In line with these results, preincubation with the unspecific NO synthase inhibitor L-NAME (0.1 mmol/L) significantly increased the EFS-contractile response in segments from both experimental groups. This increase was lower in segments from BF rats (Figure 6).

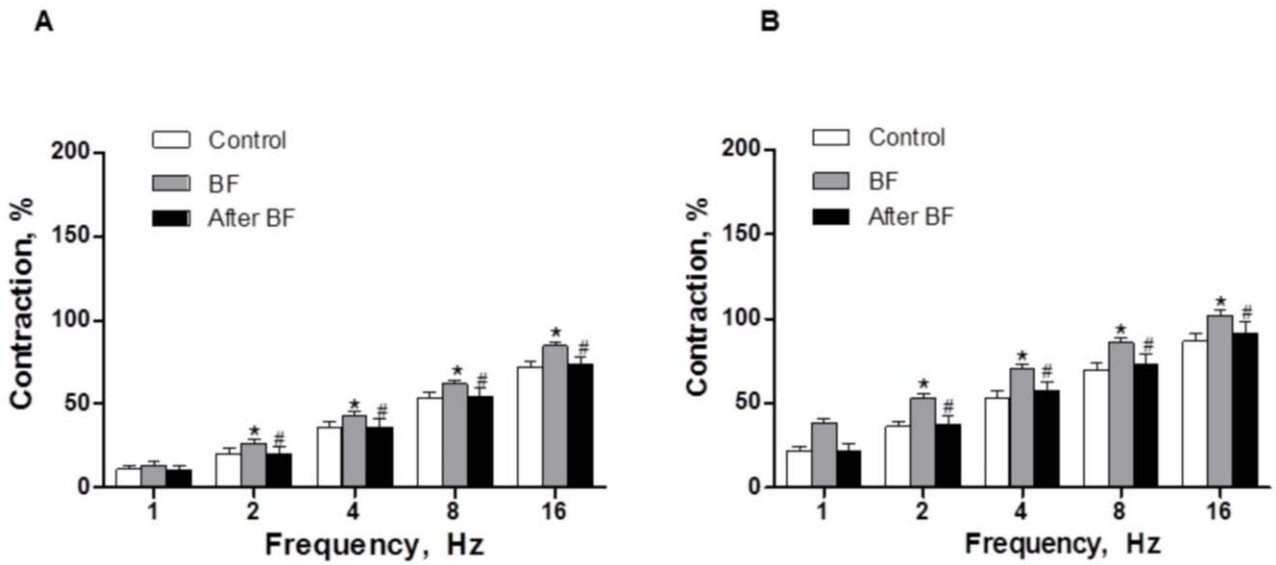

Figure 2. Vasoconstrictor response to EFS. EFS-induced vasoconstriction in endothelium-intact (A) and endothelium-removed (B) mesenteric segments from control, breast feeding (BF) and after BF rats. Results (Mean±S.E.M.) were expressed as a percentage of the initial contraction elicited by KCl. ANOVA $P<0.05$ Control vs. BF; ANOVA $P<0.05$ BF vs. After BF in both endothelium intact (A) and endothelium-removed (B) arteries. *$P<0.05$ vs. control animals at each frequency; #$P<0.05$ vs. BF group at each frequency (Bonferroni test). n = 10 animals each group.
doi:10.1371/journal.pone.0053802.g002

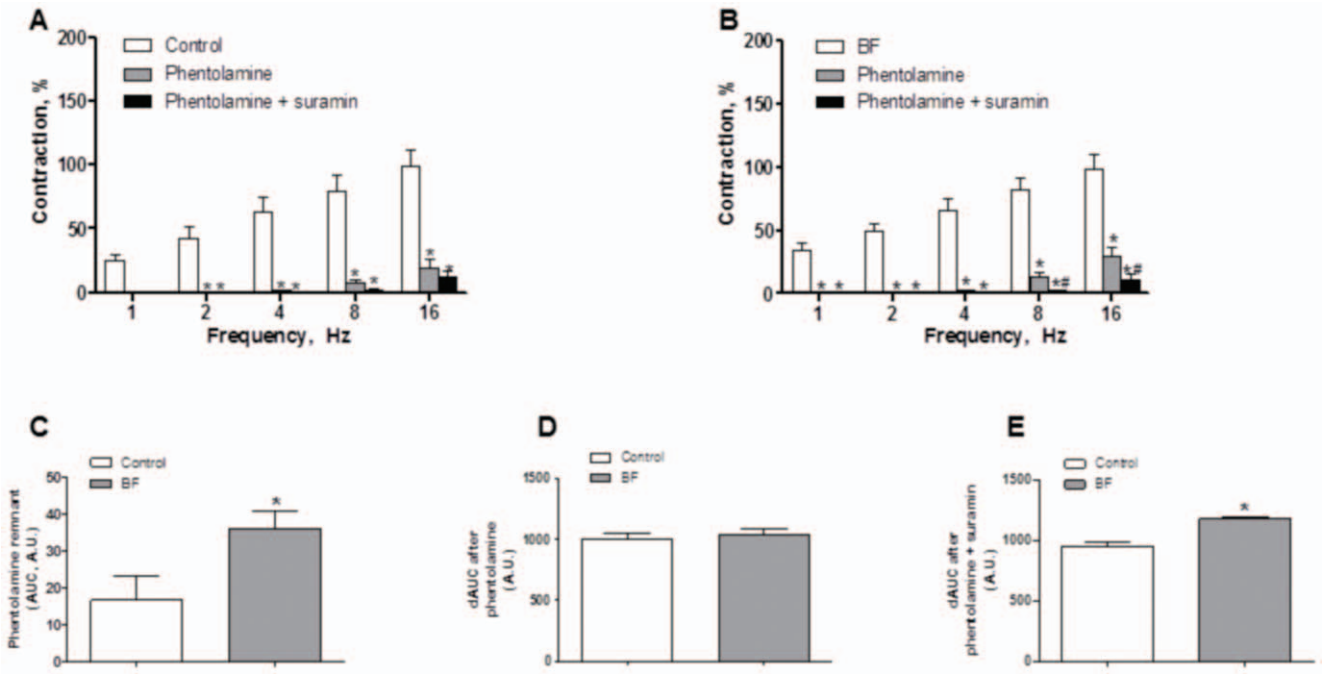

Figure 3. Effect of breast feeding on sympathetic innervation function. Effect of preincubation with 1 μmol/L phentolamine or 1 μmol/L phentolamine plus 0.1 mmol/L suramin on the vasoconstrictor response induced by EFS in endothelium-denuded mesenteric segments from control (A) and breast-feeding (BF) rats (B). Results (Mean±S.E.M.) are expressed as a percentage of the previous contraction elicited by KCl. n = 6 animals each group. ANOVA $P<0.05$ vs. conditions without phentolamine in both experimental groups. *$P<0.05$ vs. conditions without phentolamine at each frequency (Bonferroni test). ANOVA $P<0.05$ phentolamine vs. phentolamine plus suramin in both experimental groups. # $P<0.05$ phentolamine vs. phentolamine plus suramin at each frequency (Bonferroni test). (C) Representation of remnant vasoconstriction after preincubation with 0.1 μmol/L phentolamine, expressed as area under curve (AUC). *$P<0.05$ control vs. BF. Differences of area under curve (dAUC) in the absence or presence of 01 μmol/L phentolamine (D) or in the absence or presence of 1 μmol/L phentolamine plus 0.1 mmol/L suramin (E). dAUC values are expressed as arbitrary units. *$P<0.05$ control vs. BF.
doi:10.1371/journal.pone.0053802.g003

Discussion

The vasoconstrictor response to EFS is increased by breast feeding and is restored after this physiological process. This increase is endothelium-independent, and is mediated by at least two mechanisms: an increase in ATP release and a decrease in neuronal NO release.

Important differences in vascular reactivity to different agonists have been described in several physiological processes associated to changes in hormone levels. Previous results have been contradictory, since changes as well as no changes have both been reported in the vasoconstrictor response to KCl associated with modifications in sexual hormone levels [23,24,25]. In our experimental conditions the vasoconstrictor response to KCl remained unmodified by breast feeding.

When analysing the vasoconstrictor response induced by EFS in endothelium-intact segments, we observed that EFS produced a frequency-dependent contraction in segments from all experimental groups. Mesenteric arteries from breast-feeding rats showed an increased response to EFS. This increase was abolished after breast feeding, and was not attributable to changes in the intrinsic contractile machinery as was demonstrated by the similar vasoconstrictor response to KCl in all experimental groups. Endothelium removal increased vasoconstrictor response to EFS to the same extent in the three experimental groups, indicating that the modulating role of endothelium is not modified by breast feeding. The fact that ACh-induced vasodilation was not modified in any experimental group reinforces this observation. Therefore, these results indicate that the increment observed during breast feeding is due to modifications in perivascular innervation function, as was confirmed by the abolishment of EFS-induced vasoconstriction in the presence of TTX. Additionally, since both endothelial and neuronal function are not different when comparing control and after BF rats, we performed the following experiments only in mesenteric segments from control and BF animals.

Hence, this effect could be associated with changes in neurotransmitter release and/or vasomotor response to the neurotransmitter/s. In order to clarify this aspect, our next objective was to determine possible differences in the participation of sympathetic innervation between control and breast-feeding experimental groups. The participation of sympathetic innervation in splanchnic blood flow adaptation to different situations has been demonstrated previously [26]. Sympathetic innervation releases mainly NA and ATP when electrically stimulated. The results of several studies are inconclusive regarding whether or not sexual hormones affect sympathetic activity [27,28,29], but both increases and decreases have been reported during breast feeding [16,17,18]. The fact that the α-adrenoceptor antagonist phentolamine significantly diminished the vasoconstrictor response to EFS in mesenteric segments from both experimental groups confirms that this response appears to be mediated mainly by the release of NA from sympathetic nerve terminals. In our study the decreases in EFS vasoconstriction induced by phentolamine, NA release and exogenous NA-induced vasoconstriction were similar in both experimental groups, indicating the non-participation of the adrenergic component from sympathetic innervation in the increased vasoconstrictor response to EFS in breast-feeding rats.

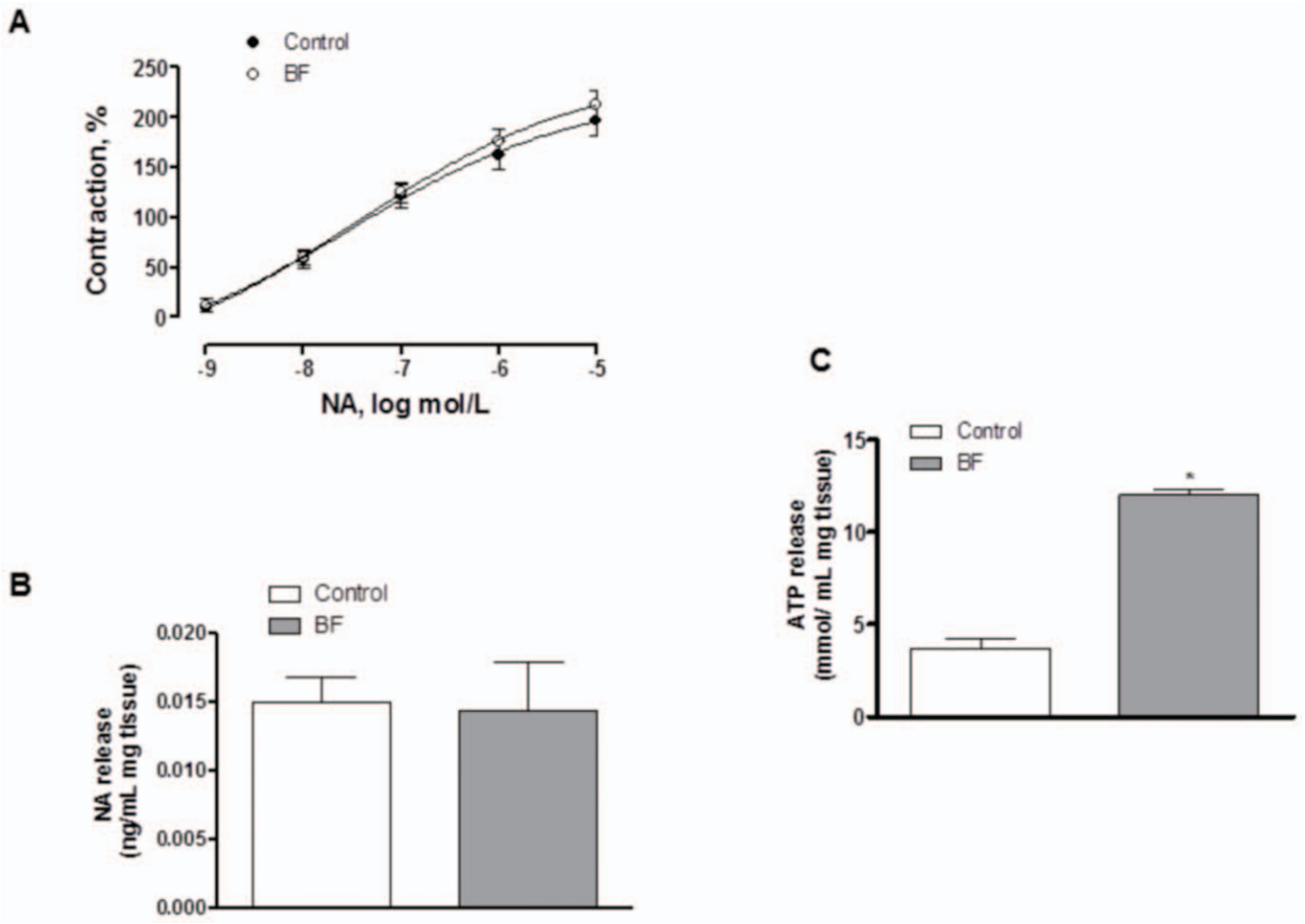

Figure 4. Effect of breast feeding on NA vasoconstriction and release, and ATP release. (A) Vasoconstrictor response to exogenous NA in segments from control and breast-feeding (BF) rats. Results (Mean±S.E.M.) are expressed as a percentage of the previous contraction elicited by KCl. n = 8 animals each group. (B) EFS-induced NA release in mesenteric segments from control and breast-feeding (BF) rats. Results (Mean±S.E.M.) are expressed as ng NA/mL mg tissue. n = 6 animals per group. (C) EFS-induced ATP release in mesenteric segments from control and breast-feeding (BF) rats. Results (Mean±S.E.M.) are expressed as mmol ATP/mL mg tissue. *P<0.05 vs control. n = 7 animals per group.
doi:10.1371/journal.pone.0053802.g004

It should also be mentioned that in both groups there was a substantial contractile response that was phentolamine-resistant. This remnant vasoconstriction was higher in segments from breast feeding animals. In mesenteric arteries we have previously demonstrated that this remnant vasoconstriction is due to ATP release from sympathetic nerve endings [30]. Based on this information, we analysed EFS-induced contraction after simultaneous preincubation with phentolamine plus the non-specific P_2 purinergic receptor antagonist suramin. In these conditions, the contractile response to EFS was reduced in both experimental groups, but the reduction was greater in segments from BF than in control rats, indicating a higher ATP participation in this response during BF. In line with these results, EFS-induced ATP release was higher in mesenteric segments from BF than control rats. Taken together, these results indicate that the greater EFS-induced vasoconstriction observed in mesenteric segments from breast-feeding rats might be due to an increase in sympathetic function through increased ATP release. However, the participation of other kinds of innervation cannot be ruled out.

Table 3. E_{max} (%) and log EC_{50} (mmo/L) values of vasoconstrictor response to NA or vasodilator response to DEA-NO in mesenteric arteries from control and BF rats.

	Control		BF	
	E_{max}	−log EC_{50}	E_{max}	–log EC_{50}
NA	226.3±55.3	7.6±0.8	242.4±45.2	7.37±0.5
DEA-NO	100.9±3.4	6.04±0.1	108.1±8.9	5.89±0.2

Results are expressed as means±S.E.M. n = 7–8 animals each group.
doi:10.1371/journal.pone.0053802.t003

We have previously reported that CGRP released from sensory innervation does not participate in the vasoconstrictor response to EFS in our experimental conditions [22,30]. However, we have also demonstrated that in several situations, such as hypertension and diabetes [11,15,33], CGRP does begin to be functional. Since sex hormones have been shown to modulate CGRP release [31], we analyzed the possibility that breast feeding might produce changes in sensory innervation function in rat mesenteric innervation. The non-modification of EFS-induced vasoconstriction obtained after preincubation with CGRP receptor antagonist CGRP (8–37) indicates that breast feeding does not modify the participation of sensory innervation-derived CGRP in vasomotor response to EFS.

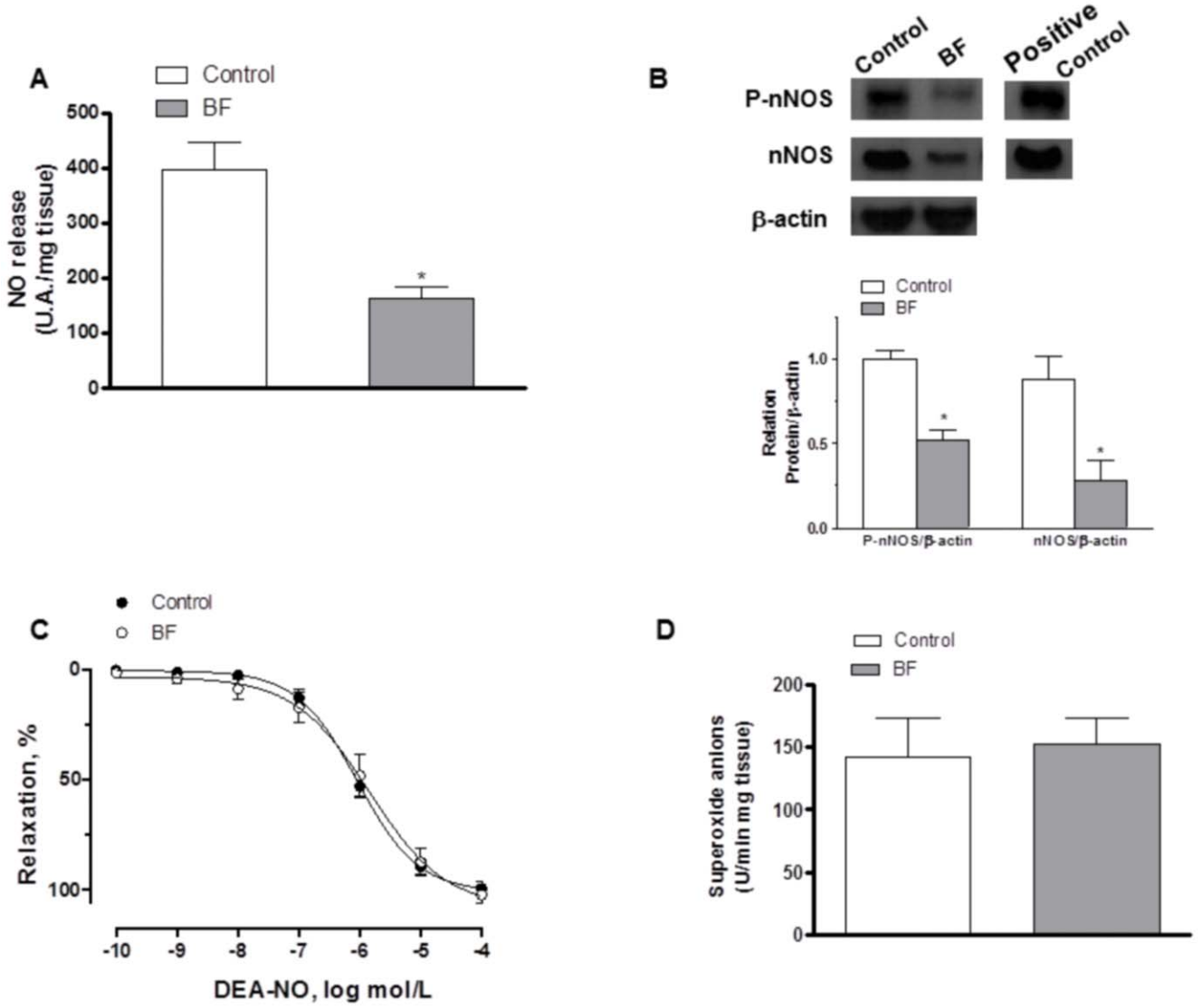

Figure 5. Effect of breast feeding on neuronal NO synthesis and vasodilation. (A) EFS-induced NO release in segments from control and breast-feeding (BF) rats. Results (Mean±S.E.M.) are expressed as arbitrary units (A.U.)/mg tissue. *P<0.05 vs. Control; n = 8 animals per group. (B) Effect of breast feeding (BF) on nNOS and P-nNOS expression. The blot is representative of four separate segments from each group. Rat brain homogenates were used as positive control. Lower panel shows relation between P-nNOS or nNOS expression and ß-actin. Results (Mean±S.E.M.) are expressed as the ratio of the signal obtained for each protein and the signal obtained for ß-actin. *P<0.05 vs. control. (C) Vasodilator response to NO donor DEA-NO in segments from control and breast-feeding (BF) rats. Results (Mean±S.E.M.) are expressed as a percentage of the previous tone elicited by exogenous NA. n = 7 animals each group. (D) Superoxide anion release in mesenteric segments from control and breast-feeding (BF) rats. Results (Mean±S.E.M.) are expressed as chemiluminiscence units (U)/min mg tissue. n = 4 animals per group.
doi:10.1371/journal.pone.0053802.g005

The participation of neuronal NO released from perivascular nitrergic innervation in the EFS-induced response has been exhaustively studied by our group [13,22,30,32]. In the experimental conditions presented in this study, the involvement of neuronal NO in the EFS-induced response is demonstrated by the increased response to EFS in segments from both experimental groups after preincubation with the unspecific NOS inhibitor L-NAME. This increase was greater in segments from breast-feeding rats, indicating that this condition could induce differences in the neuronal NO pathway. Previous studies have reported increased nitrite and nitrate levels in breast-feeding mother's serum [19], suggesting an increase in NO release, which could be originated from different sources, such as endothelium and innervation. However, in our experimental conditions, EFS-induced NO release was significantly decreased by breast feeding. NO production in neural tissue can have two sources: nNOS or iNOS [22,30,32,34]. The fact that preincubation with TTX abolished EFS-induced NO release in segments from both groups of rats, and that preincubation with the specific iNOS inhibitor 1400W did not alter NO release, confirms the neural origin and rules out the inducible origin of the NO. Since we have previously demonstrated in this vascular bed that NO released from nerve endings is synthesized through nNOS [22,30], our next objective was to determine whether this decrease was due to alterations in nNOS expression and/or activation. We observed by Western blot analysis that both nNOS and P-nNOS expression were diminished in mesenteric segments from breast-feeding rats compared to controls, indicating that the local decrease of neuronal release in

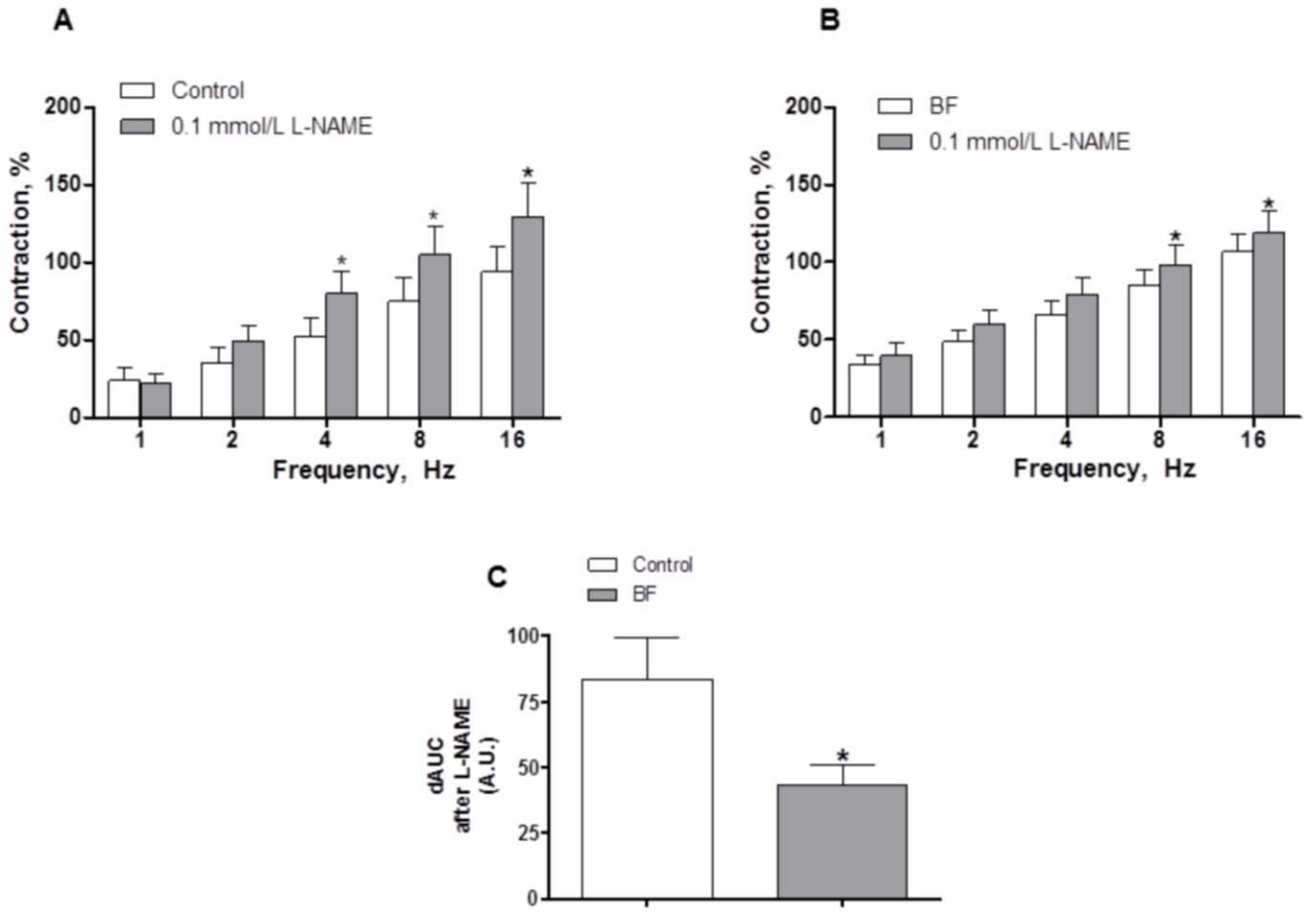

Figure 6. Effect of breast feeding on nitrergic innervation. Effect of preincubation with 0.1 mmol/L L-NAME on the vasoconstrictor response induced by EFS in mesenteric segments from control (A) and breast-feeding (BF) rats (B). Results (Mean±S.E.M.) are expressed as a percentage of the previous contraction elicited by KCl. n = 8 animals each group. ANOVA $P<0.05$ vs. conditions without L-NAME in both experimental groups. *$P<0.05$ vs. conditions without L-NAME for each frequency (Bonferroni test). (C) Differences of area under curve (dAUC) in the absence or presence of 01 μmol/L L-NAME. dAUC values are expressed as arbitrary units. *$P<0.05$.
doi:10.1371/journal.pone.0053802.g006

breast-feeding rats is caused by decreased nNOS expression and activation.

Increased ROS generation has been described specially at the end of gestation and the beginning of lactation [20]. Thus, the involvement of ROS in the vascular response cannot be ruled out, since it would alter *the* neuronal NO metabolism and consequently affect the bioavailability of neuronal NO. Superoxide anion release was similar in segments from both control and breast-feeding rats. Additionally, the vasodilator response to the NO donor DEA-NO was similar in segments from both experimental groups confirming that the diminished function of the nitrergic innervation is due to a decreased neuronal NO release and not to changes in the vasodilator response and/or metabolism of neuronal NO.

In conclusion, breast feeding increases the contractile response induced by EFS in mesenteric arteries and this increase appears to be mainly mediated by a decrease in the neuronal NO release mediated by decreased nNOS and P-nNOS expression. Additionally, sympathetic innervation function is increased through increased ATP release. These alterations might have relevance when mothers develop hypertension during the breast feeding period.

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Author Contributions

Conceived and designed the experiments: JB-R GB. Performed the experiments: JB-R ES LC. Analyzed the data: JB-R ES LC GB. Contributed reagents/materials/analysis tools: JB-R MG GB. Wrote the paper: JB-R ES LC GB.

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Artículo 3: Effect of short- and long-term portal hypertension on adrenergic, nitrergic and sensory functioning in rat mesenteric artery.

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Effect of short- and long-term portal hypertension on adrenergic, nitrergic and sensory functioning in rat mesenteric artery

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ABSTRACT

In the present study, we analysed possible alterations in adrenergic, nitrergic and sensory functioning in mesenteric arteries from rats at 1 and 21 months after partial portal vein ligation, and the mechanisms involved in these alterations, if any. For this purpose, we analysed the vasoconstrictor response to EFS (electrical field stimulation) and the effect of the α-antagonist phentolamine, the NOS (nitric oxide synthase) inhibitor L-NAME (N^G-nitro-L-arginine methyl ester) and the CGRP (calcitonin gene-related peptide) receptor antagonist CGRP-(8–37) in mesenteric segments from ST (short-term; 1 month) and LT (long-term; 21 months) SO (sham-operated) and pre-hepatic PH (portal hypertensive) rats. The vasomotor responses to NA (noradrenaline), the NO donor DEA-NO (diethylamine NONOate) and CGRP were analysed. NA, NO and CGRP releases were measured. Phospho-nNOS (neuronal NOS) expression was studied. The vasoconstrictor response to EFS was decreased in STPH animals. Phentolamine decreased this vasoconstrictor response more strongly in SO animals. Both L-NAME and CGRP-(8–37) increased vasoconstrictor response to EFS more strongly in PH than SO segments. PH did not modify vasomotor responses to NA, DEA-NO or CGRP, but it decreased NA release while increasing those of NO and CGRP. Phospho-nNOS expression was increased by PH. In LTPH, no differences were observed in vasoconstrictor response to EFS, vasomotor responses or neurotransmitter release when compared with age-matched SO animals. In conclusion, the mesenteric innervation may participate in the development of the characteristic hyperdynamic circulation observed in STPH through the joint action of decreased adrenergic influence, and increased nitrergic and sensory innervations influences. The participation of each innervation normalizes under conditions of LTPH.

INTRODUCTION

PH (portal hypertension) is a clinical syndrome defined, among other factors, by a pathologically altered splanchnic and systemic blood flow brought about by an increased vasodilation associated with endothelial factor alterations [1,2]. This increased vasodilation is also present when PH is maintained for a long time

Key words: aging, calcitonin gene-related peptide (CGRP), mesenteric innervation, nitric oxide, noradrenaline, portal hypertension.

Abbreviations: ACh, acetylcholine; CGRP, calcitonin gene-related peptide; DEA-NO, diethylamine NONOate; EFS, electrical field stimulation; HRP, horseradish peroxidase; KHS, Krebs–Henseleit solution; L-NAME, N^G-nitro-L-arginine methyl ester; LT, long-term; NA, noradrenaline; NOS, nitric oxide synthase; iNOS, inducible NOS; nNOS, neuronal NOS; PH, portal hypertension; PP, portal pressure; SBP, systolic blood pressure; SO, sham-operated; ST, short-term; TTX, tetrodotoxin.

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[3–6]. In fact, we have previously demonstrated increased endothelial prostacyclin participation in aorta vasodilation observed in LT (long-term) PH [5]. Nevertheless, vascular tone is the result of an equilibrium among several mechanisms, and the adrenergic, cholinergic, nitrergic, peptidergic and/or sensory innervations play important functional roles that depend on the vascular bed being analysed. The rich sympathetic [7], sensory [8] and nitrergic innervations [9,10] of rat mesenteric arteries modulate vascular tone by releasing the vasoconstrictor neurotransmitter NA (noradrenaline), and the vasodilator neurotransmitters CGRP (calcitonin gene-related peptide) and NO under electrical stimulation.

We have reported that different physiopathological situations, such as diabetes [11,12], hypertension [13,14], cirrhosis [15] or aging [16,17], can modify the participation of the different innervations in the vasomotor response to EFS (electrical field stimulation). In PH, and with regard to adrenergic innervation, both an increase and a decrease in vasoconstrictor response to NA have been reported [4,18–21], as well as a down-regulation of genes related to the adrenergic system and atrophy of mesenteric sympathetic innervation [22,23]. In reference to nitrergic innervation, increased involvement of neuronal NO in EFS-induced vasodilation [24] and in nNOS [neuronal NOS (nitric oxide synthase)] expression [25] has been described. Additionally, an increased CGRP participation in periarterial nerve stimulation has been suggested [24]. In cirrhotic rats, we have reported a pivotal role by CGRP in the decreased vasoconstrictor response induced by EFS, mediated by an increase in CGRP release and vasodilator response [15]. In summary, few studies have analysed the function of each innervation in PH or their integrated effect on vascular tone, whereas the evolution of their participation in a situation of LT maintenance of PH is also a question of some interest. Since PH can last for several years in humans, the aim of this study is to analyse the impact of both STPH (short-term PH) and LTPH on perivascular innervation function.

The present study was designed to analyse possible alterations in adrenergic, nitrergic and sensory function in mesenteric arteries from rats at 1 and 21 months after partial portal vein ligation, and the mechanisms involved in any alterations.

MATERIALS AND METHODS

Animals

The study was conducted in male Wistar rats (250 g; Harlan Ibérica SL), according to the European Union guidelines for ethical care of experimental animals and U.S. National Institutes of Health guidelines. Animals were fed rat chow and water *ad libitum*. They were housed in groups of 3–4 animals in a light/dark-controlled room, with an average temperature ($22 \pm 2\,^{\circ}C$) and humidity (65–70 %). Animals were randomly divided into two groups: SO (sham-operated) rats and rats with pre-hepatic PH produced by triple partial portal vein ligation. These animals were maintained for 1 month (STPH) or 21 months (LTPH).

Surgical techniques

Rats were anaesthetized by intraperitoneal injection of ketamine (100 mg/kg of body weight) and Xylacine (12 mg/kg of body weight). The surgical procedure to produce PH by triple partial ligation of the portal vein has been described previously [26]. In brief, a midline abdominal incision was made, the portal vein isolated and three ligatures, fixed on a sylactic guide, were performed in its superior, middle and inferior portions. The stenoses were calibrated by a simultaneous ligation (4-0 silk) around the portal vein and a 20-gauge blunt-tipped needle. The midline incision was closed in two layers with an absorbable suture (polyglycolic acid) and 3-0 silk. Analgesia was maintained during 24 h with Buprenorphine (0.05 mg/kg, 8 h subcutaneous). In SO rats, the procedure was the same except for ligature of the portal vein.

Systolic blood pressure was measured using the tail-cuff method before killing. Animals were anaesthetized by intraperitoneal injection of ketamine (100 mg/kg of body weight) and xylacine (12 mg/kg of body weight), a midline abdominal incision was made and PP (portal pressure) was registered and mesenteric venous vasculopathy and collateral circulation were studied. At the end of the experiments, each animal was killed by exanguination through the vena cava at 1 month (ST) or at 21 months (LT). The superior mesenteric artery was carefully dissected out, cleaned of connective tissue, cut into 4-mm-long segments and placed in KHS (Krebs–Henseleit solution; 115 mmol/l NaCl, 2.5 mmol/l $CaCl_2$, 4.6 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 25 mmol/l $NaHCO_3$, 11.1 mmol/l glucose and 0.03 mmol/l Na_2EDTA) at $4\,^{\circ}C$.

PP measurement

Splenic pulp pressure, an effective indirect measurement of PP, was measured as described previously [5,15] by inserting a 23-gauge fluid-filled needle into the spleen parenchyma. The needle was joined to a PE-50 tube, and then connected to a pressure recorder (PowerLab 200 ML 201) and a transducer (Sensonor SN-844) with a Chart V4.0 computer program (AD Instruments); the recorder was re-calibrated before each experiment.

Mesenteric venous vasculopathy and portosystemic collateral circulation study methods

The existence of high PP was confirmed by the development of mesenteric venous vasculopathy and

collateral portal-systemic circulation and was observed only in PH rats. First, a midline abdominal incision with a large bilateral subcostal extension was performed. Mesenteric venous vasculopathy, a characteristic feature of splanchnic venous congestion, manifests as distension and tortuosity of the superior mesenteric vein branches [27]. Portosystemic collateral circulation was studied by macroscopic examination of the areas in which the collateral venous circulation had developed, and the splenorenal, gastroesophageal, colorectal and hepatic hilum were carefully studied for the presence of increased collateral veins [28].

Vascular reactivity

The method used for isometric tension recording has been described in full elsewhere [10,29]. Briefly, two parallel stainless steel pins were introduced through the lumen of the vascular segment: one was fixed to the bath wall, and the other connected to a force transducer (Grass FTO3C); this was connected in turn to a model 7D Grass polygraph. For EFS experiments, segments were mounted between two platinum electrodes 0.5 cm apart and connected to a stimulator (model S44; Grass) modified to supply the appropriate current strength. Segments were suspended in an organ bath containing 5 ml of KHS at 37 °C continuously bubbled with a 95 % O_2/5 % CO_2 mixture (pH 7.4). Experiments were performed in endothelium-denuded segments to eliminate the main source of vasoactive substances, including endothelial NO. This avoided possible actions by different drugs on endothelial cells that could have led to result misinterpretation. Endothelium was removed by gently rubbing the luminal surface of the segments with a thin wooden stick. The segments were subjected to a tension of 0.5 g which was readjusted every 15 min during a 90-min equilibration period before drug administration. After this, the vessels were exposed to 75 mmol/l KCl to check their functional integrity. Endothelium removal did not alter the contractions elicited by 75 mmol/l KCl. After a washout period, the absence of vascular endothelium was proven by the inability of 10 μmol/l ACh (acetylcholine) to relax segments pre-contracted with NA (995 $\pm$ 1.29 mg).

Frequency-response curves to EFS (1, 2, 4, 8 and 16 Hz) were performed. The parameters used for EFS were 200 mA, 0.3 ms, 1–16 Hz, for 30 s with an interval of 1 min between each stimulus, the time required to recover basal tone. A washout period of at least 1 h was necessary to avoid desensitization between consecutive curves. Three successive frequency–response curves separated by 1 h intervals produced similar contractile responses. To evaluate the neural origin of the EFS-induced contractile response, the nerve impulse propagation blocker TTX (tetrodotoxin; 0.1 μmol/l) [10] was added to the bath 30 min prior to performing the second frequency–response curve.

To determine the participation of adrenergic innervation in the EFS-induced response in segments from all experimental groups, 1 μmol/l phentolamine [10], an α-adrenoceptor antagonist, was added to the bath 30 min before performing the frequency–response curve. Additionally, the vasoconstrictor response of exogenous NA (1 nmol/l to 10 μmol/l) was tested in segments from all experimental groups.

To analyse the participation of nitrergic innervation in the EFS-induced response in segments from all rat groups, 0.1 mmol/l L-NAME (N^G-nitro-L-arginine methyl ester) [10], a non-specific inhibitor of NOS, was added to the bath 30 min before performing the second frequency–response curve. To rule out the inducible origin of NO, a similar protocol was performed with the specific iNOS (inducible NOS) inhibitor 1400W (1 μmol/l) [30]. The vasodilator response to the NO donor, DEA-NO (diethylamine NONOate; 0.1 nmol/l to 0.1 mmol/l) was determined in NA-pre-contracted arteries from all experimental groups.

To study the possible participation of sensitive innervation in the EFS-induced response in segments from all experimental groups, 0.5 μmol/l CGRP-(8–37) [31], a CGRP receptor antagonist, was added to the bath 30 min before performing the second frequency–response curve. The vasodilator response to exogenous CGRP (0.1 nmol/l to 0.1 μmol/l) was analysed in NA-pre-contracted segments from all animal groups.

NA and CGRP release

Endothelium-denuded mesenteric segments from STSO, STPH, LTSO and LTPH rats were pre-incubated for 30 min in 5 ml of KHS at 37 °C and continuously gassed with a 95 % O_2/5 % CO_2 mixture (stabilization period). This was followed by two washout periods of 10 min in a bath of 0.4 ml of KHS, after which arteries were subjected to cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz at 1 min intervals, as described above.

The release of NA and CGRP was measured by using an NA research EIA (enzyme immunoassay) kit (Labor Diagnostica Nord) and a rat CGRP enzyme immunoassay kit (Spibio) respectively. The different assays were performed following manufacturers' instructions. Results are expressed as ng of NA/ml per mg of tissue and pg of CGRP/ml per mg of tissue.

NO release

After an equilibration period of 60 min in Hepes buffer (119 mmol/l NaCl, 20 mmol/l Hepes, 1.2 mmol/l $CaCl_2$, 4.6 mmol/l KCl, 1 mmol/l $MgSO_4$, 0.4 mmol/l KH_2PO_4, 5 mmol/l $NaHCO_3$, 5.5 mmol/l glucose and 0.15 mmol/l Na_2HPO_4, pH 7.4) at 37 °C, endothelium-denuded mesenteric segments from each experimental group were incubated with the fluorescent probe 4,5-diaminofluorescein (DAF-2; 2 μmol/l) for 45 min. Then, the medium was collected to measure basal NO release.

Afterwards, cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz were applied at 1 min intervals. The fluorescence of the medium was measured at room temperature (22–25 °C) using a spectrofluorimeter (LS50 with FL WINLAB Software; PerkinElmer) with $\lambda_{ex} = 492$ nm and $\lambda_{em} = 515$ nm.

The EFS-induced NO release was calculated by subtracting basal NO release from that evoked by EFS. In addition, blank samples were collected in the same way from segment-free medium in order to subtract background emission. Some assays were performed in the presence of 0.1 mmol/l L-NAME, 1 μmol/l 1400W or 0.1 μmol/l TTX to ensure the specificity of the method. The amount of released NO was expressed as arbitrary units/mg of tissue.

Western blot analysis

Endothelium-denuded mesenteric segments from all groups of rats were homogenized in a boiling buffer composed of 1 mM sodium vanadate, 1 % SDS, and 0.01 M Tris/HCl, pH 7.4. Homogenates containing 40 μg of protein were electrophoretically separated by SDS/PAGE (7.5 % gel), and then transferred on to PVDF membranes (Immun-Blot; Bio-Rad Laboratories) overnight at 4 °C and 230 mA using a Bio-Rad Mini Protean III system (Bio-Rad Laboratories) containing 25 mM Tris, 190 mM glycine, 20 % methanol and 0.05 % SDS. The membrane was blocked for 1 h at room temperature in Tris-buffered saline (100 mM Tris/HCl, pH 7.4, 0.9 %, NaCl and 0.1 % SDS) with 5 % BSA before being incubated overnight at 4 °C with a rabbit monoclonal antibody against nNOS (1:1000 dilution; Abcam) or a rabbit polyclonal antibody against phospho-nNOS (1:1000 dilution; Abcam). After washing, the membranes were incubated with anti-rabbit HRP (horseradish peroxidase)-conjugated IgG antibody (GE Healthcare). The membrane was thoroughly washed and the immunocomplexes were then detected using an enhanced HRP/luminol chemiluminescence system (ECL Plus; GE Healthcare) and subjected to autoradiography (Hyperfilm ECL, GE Healthcare). Signals on the immunoblot were quantified using a computer program (NIH Image version 1.56; National Institute of Health, Bethesda, MD, U.S.A.). The same membrane was used to determine α-actin expression, and the content of the latter was used to correct protein expression in each sample by means of a monoclonal anti α-actin antibody (1:2000 dilution; Sigma). Rat brain homogenate was used as positive control.

Drugs used

L-NA hydrochloride, ACh chloride, DEA-NONOate diammonium salt, CGRP-(8–37), rat CGRP, TTX, L-NAME hydrochloride, phentolamine, 1400W, tempol and DAF-2 were from Sigma. Stock solutions (10 mmol/l) of drugs were made in distilled water, except for NA which was dissolved in a 0.9 % NaCl/0.01 % ascorbic acid. These solutions were kept at -20 °C and appropriate dilutions were made in KHS on the day of the experiment.

Data analysis

The responses elicited by EFS or NA are expressed as a percentage of the initial contraction elicited by 75 mmol/l KCl for comparison between SO and PH rats. The relaxation induced by DEA-NO or CGRP is expressed as a percentage of the initial contraction elicited by NA. Results are given as means $\pm$ S.E.M. Statistical analysis was done by comparing the curve obtained in the presence of the different substances with the previous or control curve by means of repeated measure (two-way ANOVA). The analysis of individual points of each curve was analysed using Bonferroni post-hoc test. For the experiments on NO, CGRP and NA release, the statistical analysis was done using one-way ANOVA, followed by Newman–Keuls post-hoc test. $P < 0.05$ was considered significant.

RESULTS

Animal evolution

STPH and LTPH did not modify SBP (systolic blood pressure), although it did raise PP (Table 1). Body, spleen and liver weights were similar in both ST and LT SO and PH animals (Table 1). All rats in the STPH and LTPH groups presented portosystemic collateral circulation (pararectal, paraoesophageal and splenorenal collateral vessels).

Vascular reactivity

The response induced by 75 mmol/l KCl was similar in segments from all rat groups (STSO, 1184 ± 79.77 mg; STPH, 1010 ± 92.67 mg; LTSO: 1447 ± 96.34 mg; LTPH: 1322 ± 108.1 mg; $P > 0.05$; $n = 10$ animals each group). The contractions elicited by EFS were lower in mesenteric segments from STPH rats than those from STSO rats ($P < 0.05$; Figure 1A). EFS-induced contractions were similar in segments from LTSO and LTPH animals ($P > 0.05$; Figure 1B). Pre-incubation with the nerve impulse propagation blocker TTX (0.1 μmol/l) practically abolished EFS-induced contractions in segments from all experimental groups (Figure 2).

The contraction elicited by EFS was significantly reduced by the α-adrenoceptor antagonist, phentolamine (1 μmol/l), in segments from all rat groups (Figure 3). This decrease was greater in segments from STSO than from STPH rats. (Figures 3A and 3B, and Table 2). Phentolamine decreased EFS-induced vasoconstriction to a similar extent in segments from both LT groups (Figures 3C and 3D, and Table 2).

Table 1 Modification of body weight, SBP, spleen weight and liver weight in STSO, STPH, LTSO and LTPH rats

Results are expressed as means ± S.E.M.; $n = 10$ animals in each group. $^*P < 0.05$ compared with age-matched STSO.

Group	Body weight (g)	SBP (mmHg)	PP (mmHg)	Spleen weight/ body weight (%)	Liver weight/ body weight (%)
STSO	337.4 ± 6.6	123.4 ± 1.8	5.45 ± 0.77	0.24 ± 0.07	2.4 ± 0.1
STPH	330.2 ± 6.1	119.4 ± 1.2	9.90 ± 0.64*	0.30 ± 0.01	2.5 ± 0.06
LTSO	617.3 ± 83.5	137.1 ± 2.2	9.01 ± 1.14	0.22 ± 0.06	2.56 ± 0.31
LTPH	629.9 ± 86.4	120.5 ± 7.6	12.68 ± 1.27*	0.18 ± 0.07	2.53 ± 0.25

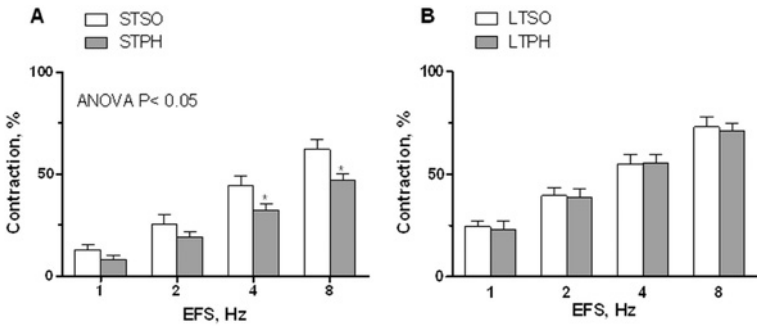

Figure 1 Isometric tension recording of the frequency–dependent contractions in denuded mesenteric artery segments from STSO and STPH rats (A), and LTSO and LTPH rats (B)

Results (means ± S.E.M.) are expressed as a percentage of tone induced by 75 mmol/l KCl; $n = 10$ animals in each group. $^*P < 0.05$ compared with control animals for each frequency (Bonferroni test).

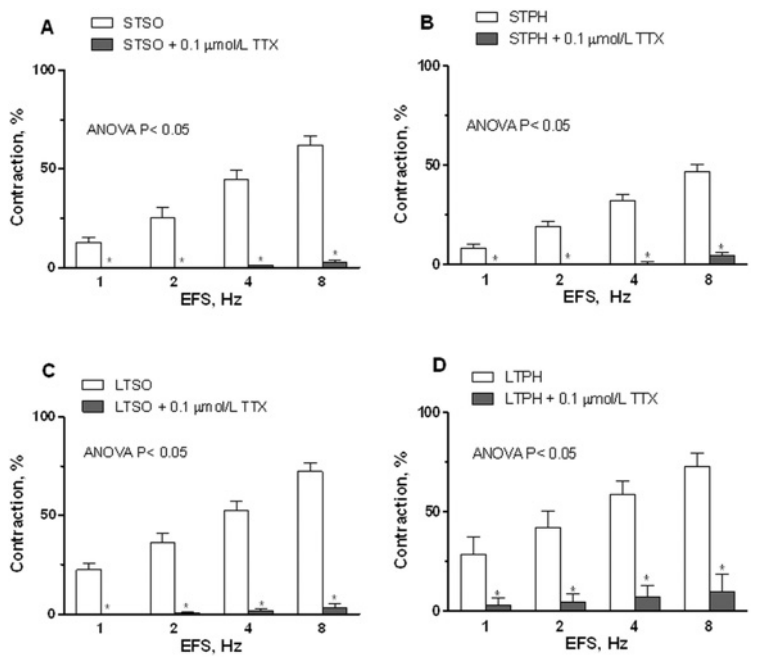

Figure 2 Effect of 0.1 μmol/L TTX on the frequency–response curves performed in endothelium-denuded mesenteric artery segments from STSO, STPH, LTSO and LTPH rats

(A and C) Results from SO animals; (B and D) results from PH rats. Results (means ± S.E.M.) are expressed as a percentage of a previous tone with 75 mmol/l KCl; $n = 10$ animals in each group. $^*P < 0.05$ compared with conditions without specific inhibitor at each frequency (Bonferroni test).

Table 2 Respective EFS inhibition/potentiation after pre-incubation with 1 μmol/l phentolamine or 0.1 mmol/l L-NAME

Results are expressed as means ± S.E.M. of the percentage inhibition/potentiation of EFS-induced contraction after pre-incubation with 1 μmol/l phentolamine or 0.1 mmol/l L-NAME in STSO, STPH, LTSO and LTPH animals; $n = 10$ animals in each group. Calculations are performed taking the control EFS-induced contraction as 100% of the contractile response. $^*P < 0.05$ compared with age-matched SO.

	EFS inhibition/potentiation (%)			
Group	1 Hz	2 Hz	4 Hz	8 Hz
STSO + phentolamine	100 ± 0.1	100 ± 0.3	97 ± 1.0	93.5 ± 2.5
STPH + phentolamine	100 ± 0.2	100 ± 0.3	79.7 ± 3.2*	71.0 ± 6.1*
LTSO + phentolamine	100 ± 0.1	97.7 ± 2.3	72.0 ± 7.1	51.3 ± 4.5
LTPH + phentolamine	100 ± 0.2	96.3 ± 3.7	69.3 ± 5.3	51.0 ± 4.4
STSO + L-NAME	38.5 ± 2.9	99.1 ± 7.4	60.4 ± 6.9	28.6 ± 3.3
STPH + L-NAME	105.4 ± 2.8*	152.5 ± 9.5*	110.3 ± 7.3*	80.5 ± 6.4*
LTSO + L-NAME	40.3 ± 3.9	96.5 ± 7.3	70.3 ± 8.1	26.4 ± 4.6
LTPH + L-NAME	39.1 ± 2.1	93.8 ± 4.9	68.5 ± 5.8	30.2 ± 3.6

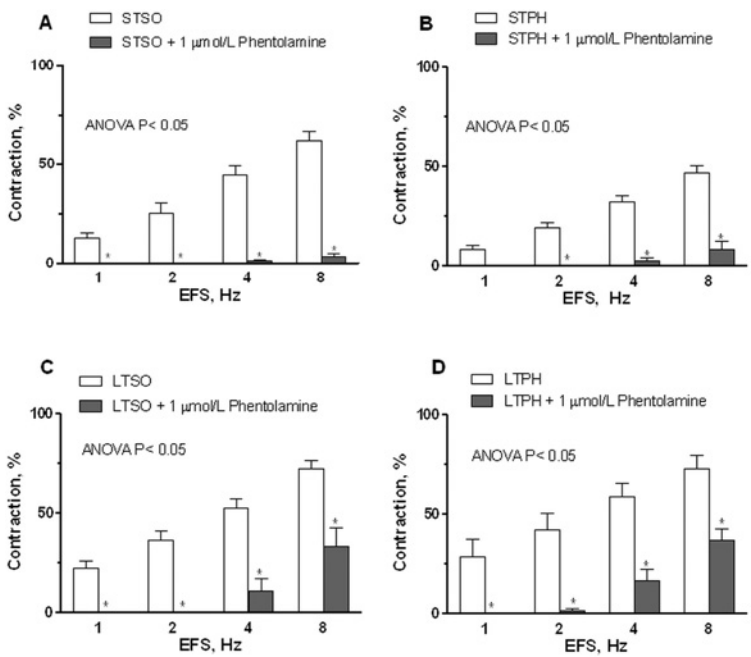

Figure 3 Effect of 1 μmol/l phentolamine on the frequency–response curves performed in endothelium-denuded mesenteric artery segments from STSO, STPH, LTSO and LTPH rats

(A and C) Results from SO animals; (B and D) results from PH rats. Results (means ± S.E.M.) are expressed as a percentage of a previous tone with 75 mmol/l KCl; $n = 10$ animals in each group. $^*P < 0.05$ compared with conditions without specific inhibitor at each frequency (Bonferroni test).

The contraction induced by EFS was significantly increased by pre-incubation with the NOS inhibitor L-NAME (0.1 mmol/l) in segments from all experimental groups (Figure 4). This increase was greater in segments from STPH compared with STSO (Figures 4A and 4B, and Table 2). L-NAME increased EFS-induced vasoconstriction to a similar extent in segments from LTSO and LTPH animals (Figures 4C and 4D). Pre-incubation with specific iNOS inhibitor 1400W (1 μmol/l) did not modify EFS-induced response in any experimental group (Figure 4).

The specific CGRP receptor antagonist CGRP-(8–37) (0.5 μmol/l) did not modify the contractile response induced by EFS in segments from STSO rats (Figure 5A), but it increased the response in segments from STPH rats (Figure 5B). Pre-incubation with CGRP-(8–37) did not modify the contractile response induced by EFS in LTSO or LTPH animals (Figures 5C and 5D).

PH did not modify vasoconstrictor responses to exogenous NA (1 nmol/l to 10 μmol/l) or vasodilator responses to NO donor DEA-NO (0.1 nmol/l to 0.1 μmol/l) and exogenous CGRP (0.1

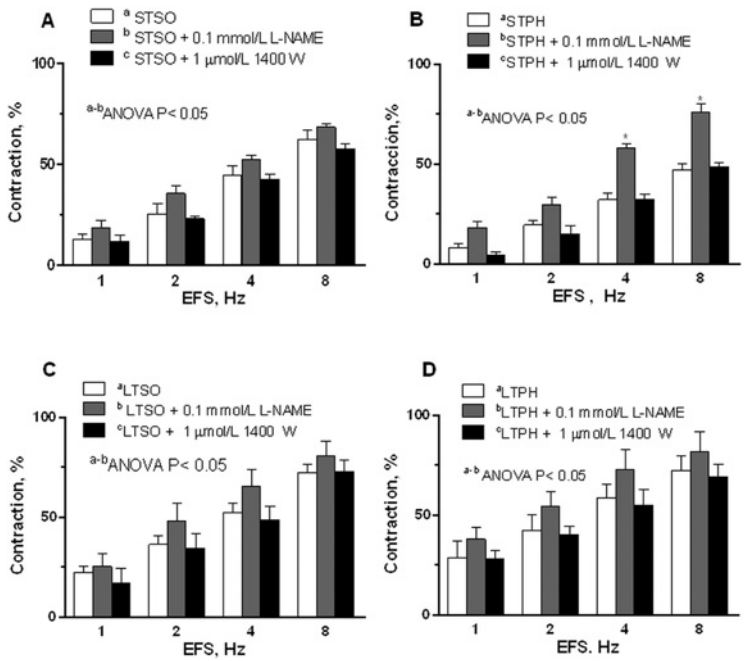

Figure 4 Effect of 0.1 mmol/l L-NAME or 1 μmol/l 1400W on the frequency–response curves performed in endothelium-denuded mesenteric artery segments from STSO, STPH, LTSO and LTPH rats

(A and C) Results from SO animals; (B and D) results from PH rats. Results (means $\pm$ S.E.M.) are expressed as a percentage of a previous tone with 75 mmol/l KCl. $n = 10$ animals in each group. $^*P < 0.05$ compared with conditions without specific inhibitor at each frequency (Bonferroni test).

nmol/l to 0.1 μmol/l) in either ST or LT animals (Figure 6).

NA release

Basal NA release was lower in mesenteric segments from STPH than from STSO rats (Figure 7A). EFS increased NA release in STSO and STPH arteries, but more strongly in segments from SO rats (Figure 7A).

Both basal and EFS-induced NA releases were similar in segments from LTSO and LTPH animals (Figure 7A).

CGRP release

In ST animals, basal CGRP release was higher in segments from PH rats than in the SO animals (Figure 7B). EFS increased CGRP levels in both groups of rats, but more so in segments from PH rats (Figure 7B).

Expression of nNOS and phospho-nNOS

A 155 kDa band was detected in nNOS analysis. nNOS expression was similar in homogenates from PH rats and SO rats at 1 month (Figure 7C). Phospho-nNOS expression was detected as a 160 kDa band, and was higher in segments from STPH rats compared with SO rats (Figure 7C).

NO release

Basal NO release was higher in mesenteric segments from STPH than from STSO rats. EFS increased NO release in both groups of rats, but more notably in segments from PH rats (Figure 7D). Both basal and EFS-induced NO releases were similar in LTSO and LTPH rats (Figure 7D). Pre-incubation with 0.1 mmol/l L-NAME or with 0.1 μmol/l TTX abolished NO release by EFS in arteries from all experimental groups, whereas 1400W (1 μmol/l) did not modify NO release (Figure 8).

DISCUSSION

Splanchnic vasodilation is the pathophysiological hallmark of the development of the hyperdynamic circulatory syndrome [18,32,33] in PH. This splanchnic vasodilation has been described to be mainly due to alterations in endothelial factors, the participation of which changes over time [3–5]. Despite the important role of perivascular innervation few studies have been performed on the possibility that alterations occur in the adrenergic, nitrergic and sensory innervations, and their integrated effects on mesenteric vascular tone. Thus, the objective of the present study was to determine whether STPH and LTPH could modify the potential functional influence of the different types of mesenteric innervation.

The vasoconstrictor response to KCl, an indicator of smooth muscle functional integrity, was not modified by the development of STPH or LTPH, in contrast with previous reports. This apparent contradiction may reflect the different experimental models used [34,35].

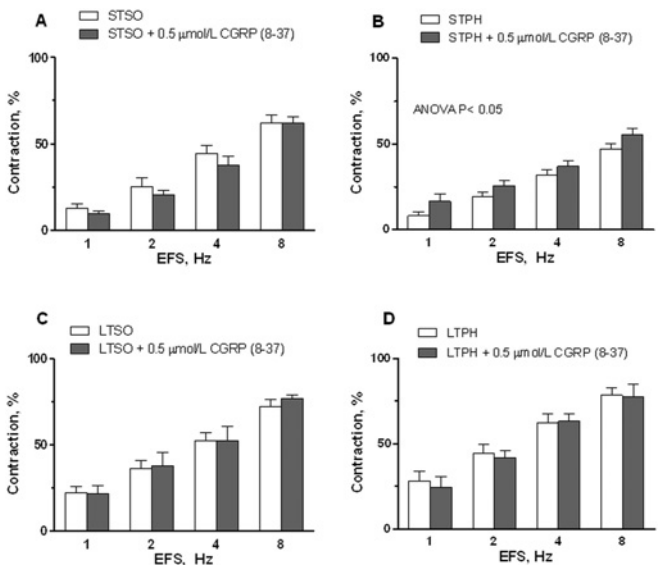

Figure 5 Effect of 0.5 μmol/l CGRP-(8–37) on the frequency–response curves performed in endothelium-denuded mesenteric artery segments from STSO, STPH, LTSO and LTPH rats

(A and C) Results from SO animals; (B and D) results from PH rats. Results (means $\pm$ S.E.M.) are expressed as a percentage of a previous tone with 75 mmol/l KCl. $n = 10$ animals each group.

Our results show that EFS induced a contractile responses in endothelium-denuded mesenteric segments from all experimental groups. This vasoconstrictor response elicited by EFS was practically abolished by the blocker for nerve impulse propagation TTX, demonstrating that the response is due to the release of neurotransmitters from the nerve endings.

Short-term PH

The operated rats had portal hyperpressure and developed systemic collateral vessels, thus confirming the validity of triple partial portal vein ligation as an experimental model to establish PH [27,28].

In STPH rats, the level of vasoconstrictor response to EFS was lower than in SO animals. This diminished vasoconstrictor response suggests neural modifications associated with PH. This effect could be associated with changes in the release of and/or response to the different neurotransmitters.

The participation of adrenergic innervation in the EFS-induced vasoconstriction was analysed by incubation with the α-adrenoceptor blocker phentolamine. The vasoconstrictor response was significantly reduced by phentolamine in STSO and STPH rats, confirming that this response is at least mediated by NA release from adrenergic nerve terminals and the subsequent activation of α-adrenoceptors. The decrease was lower in segments from PH animals, indicating a lower participation of adrenergic innervation in these animals. Our next objective was to analyse whether the decreased adrenergic participation in STPH was associated with a decrease in NA release and/or vasoconstrictor response to NA. In our experimental conditions, concentration-response curves to exogenous NA were similar in segments from both groups of rats, contrasting with previous studies reporting both an increase and a decrease of vasoconstrictor response to NA [4,18–21]; meanwhile, both basal and EFS-induced NA releases were diminished in mesenteric segments from PH rats, as also reported with reference to circulating NA [18,19]. Therefore these results explain the decrease of adrenergic function that results in the diminished vasoconstrictor response to EFS observed in STPH.

Although the diminished adrenergic function could, by itself, explain the decreased EFS vasoconstriction, the participation of other neural components cannot be ruled out. The NO released from nitrergic innervation in EFS has been demonstrated to have a vasodilator role contributing to the vasomotor response to EFS [10–14]. In vascular reactivity experiments, pre-incubation with 7-NI (7-nitroindazole) decreases vasoconstrictor response to NA, making the analysis of EFS-induced contractions very complex [36,37]. For that reason, we used the non-selective NOS inhibitor L-NAME in vascular reactivity experiments. EFS-induced vasoconstriction was increased in both experimental groups by pre-incubation with L-NAME. This increase was higher in mesenteric segments from PH than in SO rats, suggesting that the participation of nitrergic innervation is increased in PH; this could be the result of increased NO release and/or altered smooth muscle cell sensitivity to NO. The fact that the vasodilator response to the NO analogue

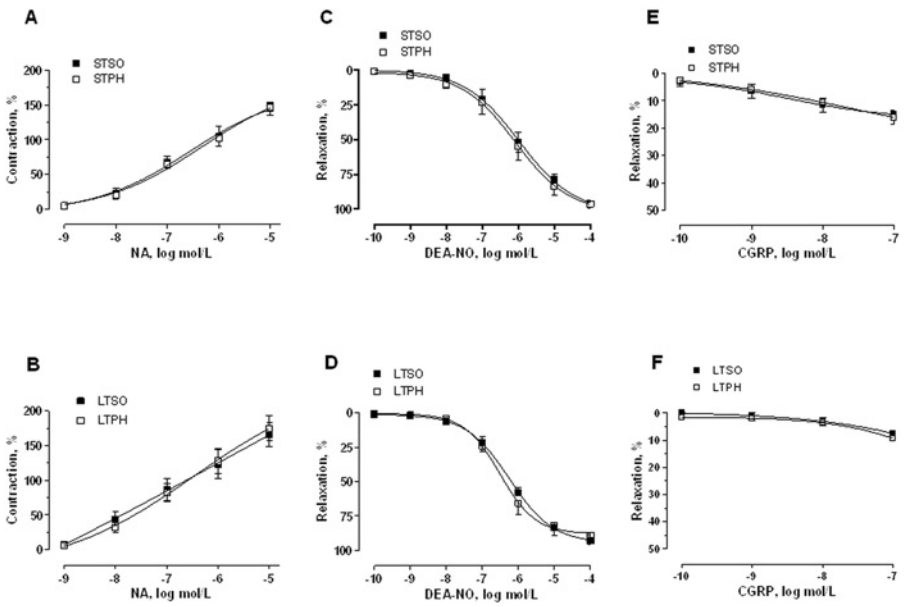

Figure 6 Vasodilator response to exogenous NA, the NO donor DEA-NO and exogenous CGRP

(A and B) Vasodilator response to exogenous NA in segments from STSO, STPH, LTSO and LTPH rats. Results (mean ± S.E.M.) are expressed as a percentage of a previous tone with 75 mmol/l KCl; $n = 8$ animals each group. (C and D) Vasodilator response to NO donor DEA-NO in segments from STSO, STPH, LTSO and LTPH rats. Results (means ± S.E.M.) are expressed as a percentage of the initial contraction elicited by exogenous NA; $n = 7$ animals in each group. (E and F) Vasodilator response to exogenous CGRP in segments from STSO, STPH, LTSO and LTPH rats. Results (means ± S.E.M.) are expressed as a percentage of the initial contraction elicited by exogenous NA; $n = 5$ animals each group.

DEA-NO was similar in both experimental groups rules out differences in smooth muscle sensitivity to NO. However, we showed directly that both basal and EFS-induced NO release were increased in PH compared with SO rats, in agreement with Jurzic et al. [25]. This augmented NO release is abolished by TTX, thus confirming the neural origin of the NO, and indicating that neuronal NO also participates in the effect of PH. Production of NO in neural tissue can have two sources: nNOS and iNOS [36–38]. However, in the present study, the specific iNOS inhibitor 1400W did not change either the vasoconstrictor response to EFS or the NO release in segments from the two experimental groups, ruling out the participation of this inducible isoform and suggesting that NO release was, indeed, dependent on nNOS expression and/or activity. We found that nNOS protein expression was not modified by PH, contrasting with previous reports [25,39], whereas the active form phospho-nNOS was increased in PH, suggesting that PH had increased nNOS activity.

Once the participation of both adrenergic and nitrergic innervation in the diminished response to EFS in segments from PH rats had been demonstrated, we studied the possible role of sensory innervation in this vasomotor response. We have previously reported that in this strain sensory innervation does not participate in vasomotor response induced by EFS in control animals, but that the participation of this innervation is increased in several pathological conditions such as hypertension, cirrhosis and diabetes [12,15,40,41]. In the present study, we observed that pre-incubation with the CGRP antagonist CGRP-(8–37) did not modify the vasoconstrictor response to EFS in mesenteric rings from SO rats, confirming CGRP non-participation in vasoconstriction in healthy Wistar rats [10,36,42]. However, CGRP-(8–37) increased the vasoconstrictor response to EFS in segments from PH rats. These results indicate that sensory innervation participates in the vasomotor response to EFS in PH rats, indicating a role for sensory innervation in the decreased vasoconstrictor response to EFS in STPH. Thus our next objective was to analyse whether this participation was associated with an increase in the CGRP release and/or vasodilatory response. The vasodilator response to exogenous CGRP was similar in segments from both experimental groups, ruling out differences in sensitivity to CGRP due to PH, meanwhile both basal and EFS-induced CGRP releases were higher in mesenteric segments from PH rats, indicating that sensory innervation also contributed to the diminished vasoconstrictor response to EFS in PH through an increase in CGRP release.

Altogether, these results confirm that in STPH the EFS-induced vasoconstrictor response in mesenteric arteries is decreased as a result of decreased adrenergic innervation function and increased sensory and nitrergic innervation function.

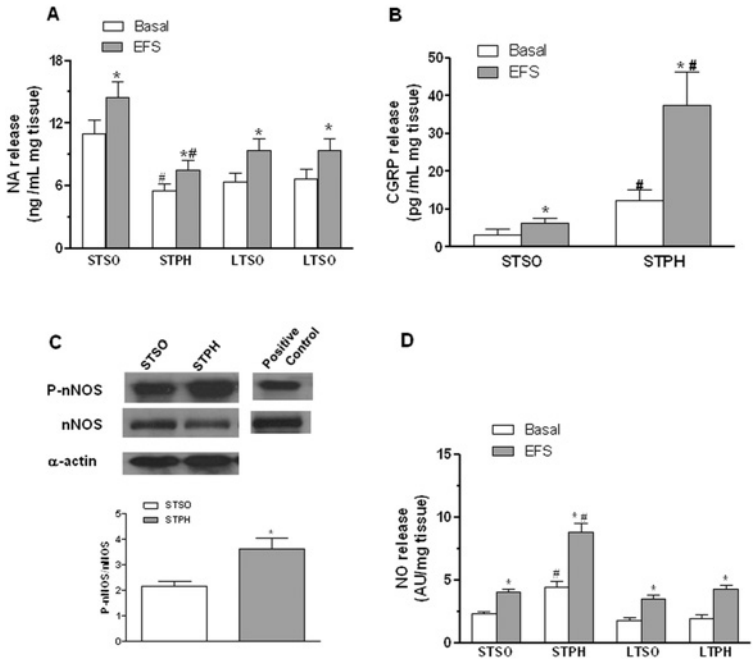

Figure 7 Neurotransmitter release and Western blot analysis

(A) Effect of STPH and LTPH on basal and EFS-induced NA release in endothelium-denuded mesenteric segments; $n = 6$ animals each group. Results are expressed as ng of NA/ml per mg of tissue. $^{*}P < 0.05$ compared with basal; $\#P < 0.05$ compared with SO. (B) Effect of STPH on basal and EFS-induced CGRP release in mesenteric segments. Results are expressed as pg of CGRP/ml per mg of tissue; $n = 6$ animals. $^{*}P < 0.05$ compared with basal; $\#P < 0.05$ compared with SO. (C) Western blot for phospho-nNOS and nNOS expression in endothelium-denuded mesenteric artery segments from STSO and STPH rats. The Figure is representative of preparations from six rats in each group. Lower panel shows relation between densitometric analysis for phospho-nNOS compared with nNOS expression. (D) Effect of STPH and LTPH on basal and EFS-induced NO release in endothelium-denuded mesenteric segments; $n = 6$ animals in each group. Results are expressed as arbitrary units (A.U.)/mg of tissue. $^{*}P < 0.05$ compared with basal; $\#P < 0.05$ compared with STSO.

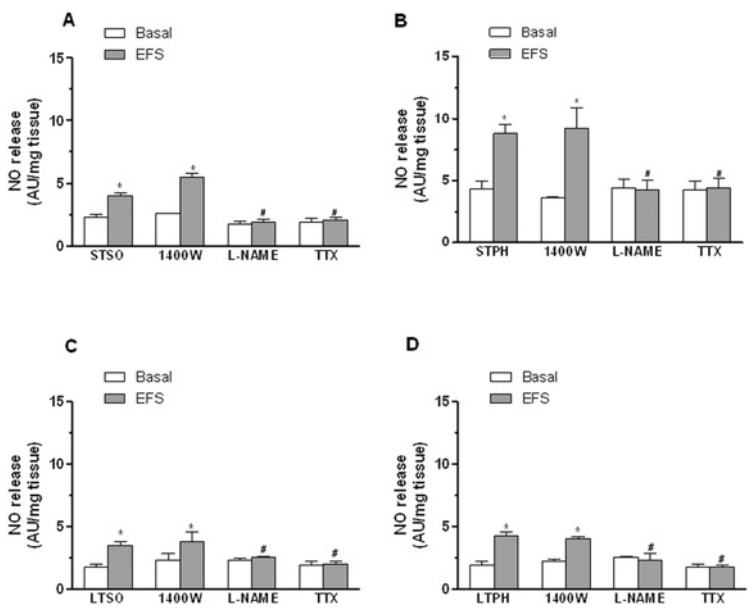

Figure 8 Effect of 1 μmol/l 1400W, 0.1 mmol/l L-NAME or 0.1 μmol/l TTX on basal and EFS-induced NO release in endothelium-denuded mesenteric segments from (A) STSO, (B) STPH, (C) LTSO and (D) LTPH rats

Results are expressed as arbitrarial units (A.U.)/ mg of tissue; $n = 6$ animals in each group. $^{*}P < 0.05$ compared with basal; $\#P < 0.05$ compared with conditions without specific inhibitor.

Long-term PH

The operated rats presented portal hyperpressure and had systemic collateral vessels at 21 months after portal vein ligation. The persistence in these changes related to PH indicates that triple partial portal vein ligation in the rat is an appropriate experimental model that maintains its validity even during very late stages, making it possible to establish a correlation with human PH, as previously suggested [5].

In later stages, an increase in splanchnic blood flow leads to the hyperdynamic circulation state, which in turn contributes to the maintenance and aggravation of many of the complications of PH [43]. In this sense, we have reported previously that maintenance of PH for 21 months also maintains the changes in endothelial function that result from increased prostacyclin release, and contributes to maintaining the increased vasodilator response to ACh [5]. Consequently, our next aim was to determine whether the different innervations also play a role in the hyperdynamic circulation present in LTPH.

The vasoconstrictor response to EFS is stronger in LTSO rats than in STSO animals as a consequence of aging, as we have previously reported [16]. In contrast with the decrease observed in STPH rats, the vasoconstrictor response to EFS is not modified in LTPH rats compared with their age-matched SO animals. This result indicates a reversion of the decreased vasoconstrictor effect on innervation in STPH, and contrasts with its effect on endothelial function [5]. However, a rearrangement of the participation of each kind of innervation cannot be ruled out.

In both experimental groups, phentolamine reduced the vasoconstrictor response to EFS to a similar extent, and both the vasoconstrictor response to NA and NA release were similar in both SO and PH rats, indicating a restored function of the adrenergic innervation at 21 months. EFS-induced vasoconstriction was similarly increased by L-NAME in both experimental groups. Additionally, neither the basal and the EFS-induced NO release nor vasodilator response to the NO analogue DEA-NO were modified by LTPH. These results indicate that the participation of nitrergic innervation is also restored in LTPH. The fact that pre-incubation with CGRP-(8–37) did not alter vasoconstrictor response to EFS in SO confirms the non-participation of CGRP in aging [16], and indicates that, in contrast to STPH, the influence of sensory innervation is attenuated in LTPH.

Taken together, the present results indicate that the innervations of mesenteric arterial vessels may well participate in the development of the characteristic hyperdynamic circulation observed in STPH through the combined actions of a decrease in the influence of the adrenergic innervation and an increase in the influences of the nitrergic and sensory innervations. By contrast, we suggest that the influences of each type of innervation normalizes under the conditions of LTPH.

AUTHOR CONTRIBUTION

Esther Sastre performed some of the experiments and statistical analyses. Gloria Balfagón performed some of the statistical analyses, discussed the results and wrote the paper. Elena Revuelta-López and Maria-Paz Nava performed the surgical techniques and the PP measurements. Maria-Ángeles Aller and Jaime Arias collaborated in the discussion of the results. Javier Blanco-Rivero performed some of the experiments and statistical analyses, discussed the results and wrote the paper

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Artículo 4: Alterations in perivascular innervation function in mesenteric arteries from offspring of diabetic rats.

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Alterations in perivascular innervation function in mesenteric arteries from offspring of diabetic rats

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BACKGROUND AND PURPOSE

We have reported that exposure to a diabetic intrauterine environment during pregnancy increases blood pressure in adult offspring, but the mechanisms involved are not completely understood. This study was designed to analyse a possible role of perivascular sympathetic and nitrergic innervation in the superior mesenteric artery (SMA) in this effect.

EXPERIMENTAL APPROACH

Diabetes was induced in pregnant Wistar rats by a single injection of streptozotocin. Endothelium-denuded vascular rings from the offspring of control (O-CR) and diabetic rats (O-DR) were used. Vasomotor responses to electrical field stimulation (EFS), NA and the NO donor DEA-NO were studied. The expressions of neuronal NOS (nNOS) and phospho-nNOS (P-nNOS) and release of NA, ATP and NO were determined. Sympathetic and nitrergic nerve densities were analysed by immunofluorescence.

KEY RESULTS

Blood pressure was higher in O-DR animals. EFS-induced vasoconstriction was greater in O-DR animals. This response was decreased by phentolamine more in O-DR animals than their controls. L-NAME increased EFS-induced vasoconstriction more strongly in O-DR than in O-CR segments. Vasomotor responses to NA or DEA-NO were not modified. NA, ATP and NO release was increased in segments from O-DR. nNOS expression was not modified, whereas P-nNOS expression was increased in O-DR. Sympathetic and nitrergic nerve densities were similar in both experimental groups.

CONCLUSIONS AND IMPLICATIONS

The activity of sympathetic and nitrergic neurons is increased in SMA from O-DR animals. The net effect is an increase in EFS-induced contractions in these animals. These effects may contribute to the increased blood pressure observed in the offspring of diabetic rats.

Abbreviations

DAF-2, 4, 5-diaminofluorescein; DEA-NO, diethylamine nonoate diammonium salt; EFS, electrical field stimulation; O-CR, control offspring; O-DR, diabetic offspring; SMA, superior mesenteric artery

Tables of Links

TARGETS	
GPCRs[a]	**Enzymes**[c]
α_2 adrenoceptor	Dopamine β-hydroxylase (DBH)
P2Y receptors	eNOS
Ligand-gated ion channels[b]	iNOS
P2X4 receptor	nNOS

LIGANDS	
1400 W	NA
ACh	Nitric oxide (NO)
ATP	Phentolamine
CGRP	Suramin
CGRP (8-37)	TTX
L-NAME	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013).

Introduction

A growing body of evidence during the last decade suggests that adverse environmental conditions during crucial periods of development may cause profound structural and biochemical changes in the body, resulting in modifications that, in later life, can become threats to health (Barker, 2004; Visentin *et al.*, 2014). In line with this, maternal diabetes is a common medical complication in pregnancy that has been rapidly increasing worldwide. Both epidemiological investigations and animal studies have shown that intrauterine exposure to a hyperglycaemic environment can affect embryonic and fetal life, predisposing the offspring to develop metabolic disorders, including diabetes and hypertension in adult life (Simeoni and Barker, 2009).

The molecular mechanism underlying the association between diabetes during pregnancy and elevated blood pressure in the offspring remains unclear, and the relatively few existing studies have rarely address vascular function. Holemans *et al.* (1999) showed a reduced relaxation to endothelium-dependent dilators and enhanced constriction to NA in small mesenteric arteries in the offspring of diabetic rats. Rocha *et al.* (2005), in this same model, found early life hypertension with renal function impairment and decreased endothelium-dependent vasodilatation in mesenteric microvessels. We ourselves have recently demonstrated an enhanced formation of vasoconstrictor prostanoids in mesenteric resistance arteries from the offspring of diabetic rats, which contributes to a hyper-reactivity to NA that may participate in their hypertension (Ramos-Alves *et al.*, 2012a,b).

It is well known that vascular tone is determined by an equilibrium among several mechanisms in which perivascular innervation plays an important role. Tone regulation involves sympathetic, cholinergic, nitrergic, peptidergic and/or sensory neurons that are specific to the vascular bed under consideration (Loesch, 2002; Sastre *et al.*, 2010). It is well known that the splanchnic circulation makes an important haemodynamic contribution to the early development of hypertension, and an increase in vascular resistance is apparent in various splanchnic organs (Meininger *et al.*, 1985). The superior mesenteric artery (SMA) regulates around 20% of total blood flow; thus, changes in mesenteric vascular tone are involved in total peripheral resistance. The application of electrical field stimulation (EFS) produces a vasoconstrictor response that is the integrated result of the release of different neurotransmitters, including the sympathetic vasoconstrictors NA and ATP, NO from nitrergic neurons and CGRP from sensory nerves (Kawasaki *et al.*, 1988; Li and Duckles, 1992; Marín and Balfagón, 1998; Blanco-Rivero *et al.*, 2013b). Alterations in the functional role of these components in rat mesenteric artery have been associated with several experimental and pathophysiological conditions including hypertension, a high fat diet intake, ageing and portal hypertension (Marín *et al.*, 2000; Ferrer *et al.*, 2003; Blanco-Rivero *et al.*, 2011a; Sastre *et al.*, 2012a). Additionally, we have previously reported that diabetes alters the function of perivascular neurons in rat mesenteric arteries (Ferrer *et al.*, 2000; del Campo *et al.*, 2011). These findings suggest that perivascular neurons in the mesenteric circulation have an important influence on the development of several kinds of hypertension, such as angiotensin II-salt hypertension (Osborn and Fink, 2010), mild DOCA-salt hypertension (Kandlikar and Fink, 2011) and renovascular hypertension (Koyama *et al.*, 2010).

Taking into account this information, we considered it relevant to investigate whether the development of maternal diabetes during pregnancy could lead to alterations in the perivascular neurons, which could affect vascular tone in the adult offspring of diabetic rats. For this purpose, we analysed the possible functional changes in perivascular neurons in mesenteric arteries from the offspring of diabetic rats, evaluating any alterations in the activity of sympathetic, nitrergic or sensory neurons, as well as the mechanisms involved.

Methods

Ethics statement

All animals were obtained from the Animal Quarters of the Universidad Autónoma de Madrid and housed in the animal facility of the Universidad Autónoma de Madrid (registration number EX-021U) in accordance with guidelines 609/86 of the E.E.C., R.D. 233/88 of the Ministerio de Agricultura, Pesca y Alimentación of Spain, and Guide for the Care and Use of Laboratory Animals published by the United States National

Institute of Health (NIH publication no. 85.23, revised 1985). The experimental protocol was approved by the Ethics Committee of the Universidad Autónoma de Madrid. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Animals

Virgin female Wistar rats were kept in cages with male rats in a 3:1 ratio for mating. Evidence of copulation was confirmed by the presence of sperm in a vaginal smear 24 h after which was considered to be day 1 of gestation. Seven days after the onset of pregnancy, diabetes was induced by a single injection of streptozotocin (50 mg·kg^{-1}, i.p.), as described previously (Wichi *et al.*, 2005; Ramos-Alves *et al.*, 2012a,b). Control animals received an equal volume of vehicle (citrate buffer). Blood glucose was measured 7 days after injecting either streptozotocin or citrate buffer to confirm whether or not diabetes had been induced; only rats with severe hyperglycaemia above 2 g·L^{-1} were used. During lactation, the offspring were restricted to six animals per rat. This study used only 6-month-old male offspring from diabetic mothers (O-DR) and control offspring (O-CR). Rats were weighed and killed by CO_2 inhalation followed by decapitation; the SMA was carefully dissected out, placed in Krebs–Henseleit solution (KHS, in mmol·L^{-1}: NaCl 115, $CaCl_2$ 2.5, KCl 4.6, KH_2PO_4 1.2, $MgSO_4$ 0.7, H_2O 1.2, $NaHCO_3$ 25, glucose 11.1, Na_2 EDTA 0.03) at 4°C, cleaned of perivascular fat and connective tissue and divided into 2 mm long segments using a micrometre eyepiece mounted on a Euromex Holland binocular lens. Some samples were immediately frozen in liquid nitrogen and stored at –70°C. Additionally, some segments were mounted on 100 µm wires in a small vessel myograph to measure internal diameter (1–1.2 mm in diameter; Simonsen *et al.*, 1999). No differences were found between experimental groups.

Glucose tolerance and insulin sensitivity

The oral glucose tolerance test was performed according to a standard protocol. After a 10 h fast, a single oral dose (2 g·kg^{-1} of body weight) of glucose was delivered. Blood glucose was then measured from the tail vein just before and at 30, 60, 90 and 120 min after glucose injection using test strips and reader (ACCU-CHEK®, Roche Diagnostics). After 48 h, animals were subjected to a new 10 h fast for assessment of insulin sensitivity by an insulin tolerance test. For this, regular insulin was administered i.p. at a dose of 1.5 U·kg^{-1} body weight. Blood glucose was determined before and at 15, 30, 45 and 60 min after insulin administration.

Arterial blood pressure measurement

The animals were anaesthetized with a mixture of ketamine, xylazine and acetylpromazine (64.9, 3.2 and 0.78 mg·kg^{-1}, respectively, i.p.). The right carotid artery was cannulated with a polyethylene catheter (PE-50) filled with heparin-containing saline. After 24 h, mean arterial pressure was measured in conscious animals by a pressure transducer (model MLT844; ADInstruments Pty Ltd, Castle Hill, New South Wales, Australia) and recorded using an interface and software for computer acquisition (ADInstruments Pty Ltd). Heart rate was calculated from the pressure signal recorded by a pressure transducer and connected to a computerized measuring programme (LabChat v7, ADInstruments Pty Ltd).

Serum biochemical parameters

Serum concentrations of total cholesterol, triglycerides and HDL cholesterol were determined using specific quantitative enzyme assays (Vitros Chemistry Products) and measured with a colorimetric spectrophotometer (Vitros Fusion 5.1 FS Chemistry System, Ortho-Clinical). The assays were performed following the manufacturer's instructions. Results are expressed as mg·L^{-1}. Serum concentrations of insulin were analysed using the Rat/Mouse Insulin ELISA Kit (EMD Millipore Corporation, Billerica, MA, USA). The assay was performed following the manufacturer's instructions. Results are expressed as ng insulin·mL^{-1}.

Vascular reactivity study

The method used for isometric tension recording has been described in full elsewhere (Nielsen and Owman, 1971; Marín and Balfagón, 1998). Briefly, two parallel stainless steel pins were introduced through the lumen of the vascular segment: one was fixed to the bath wall and the other connected to a force transducer (Grass FTO3C, Quincy, MA, USA); this, in turn, was connected to a model 7D Grass polygraph. For EFS experiments, segments were mounted between two platinum electrodes 0.5 cm apart and connected to a stimulator (Grass, model S44) modified to supply the appropriate current strength. Segments were suspended in an organ bath containing 5 mL of KHS at 37°C continuously bubbled with a 95% O_2 to 5% CO_2 mixture (pH 7.4). Some experiments were performed in endothelium-denuded segments to eliminate the main source of vasoactive substances, including endothelial NO. This avoided possible actions by different drugs on endothelial cells that could have led to a misinterpretation of the results. The endothelium was removed by gently rubbing the luminal surface of the segments with a thin wooden stick. The segments were subjected to a tension of 4.95 mN, which was readjusted every 15 min during a 90 min equilibration period before drug administration. After this, the vessels were exposed to 75 mmol·L^{-1} KCl to check their functional integrity. Endothelium removal did not alter the contractions elicited by KCl. After a washout period, the presence/absence of vascular endothelium was tested by the ability of 10 µmol·L^{-1} ACh to relax segments precontracted with 1 µmol·L^{-1} NA. We consider as endothelium-denuded those arteries that were unable to relax to ACh.

Vasodilator responses to ACh (0.1 nmol·L^{-1} to 10 mmol·L^{-1}) were obtained in endothelium-intact arteries from all experimental groups. Frequency–response curves to EFS (1, 2, 4, 8 and 16 Hz, a range considered to reproduce physiological situations) were performed in endothelium-intact and endothelium-denuded mesenteric segments from all experimental groups. The parameters used for EFS were 200 mA, 0.3 ms, 1–16 Hz, for 30 s with an interval of 1 min between each stimulus, the time required to recover basal tone. A washout period of at least 1 h was necessary to avoid desensitization between consecutive curves. Two successive frequency–response curves separated by 1 h intervals produced similar contractile responses. To evaluate the neural

origin of the EFS-induced contractile response, the nerve impulse propagation blocker, tetrodotoxin (TTX; 0.1 mmol·L^{-1}), was added to the bath 30 min before the second frequency–response curve was obtained.

To determine the participation of sympathetic neurons in the EFS-induced response in endothelium-denuded segments from O-CR and O-DR rats, 1 μmol·L^{-1} phentolamine, an α-adrenoceptor antagonist, or phentolamine plus 0.1 mmol·L^{-1} suramin, a non-specific purine receptor antagonist, was added to the bath 30 min before performing the frequency–response curve. Additionally, the vasoconstrictor response to exogenous NA (1 nmol·L^{-1} to 10 mmol·L^{-1}) was tested in segments from both experimental groups.

To study the possible participation of sensory neurons in the EFS-induced response in endothelium-denuded segments from O-CR and O-DR rats, 0.5 μmol·L^{-1} CGRP (8–37), a CGRP receptor antagonist, was added to the bath 30 min before performing the second frequency–response curve.

To analyse the participation of NO in the EFS-induced response in endothelium-denuded segments from O-CR and O-DR rats, 0.1 mmol·L^{-1} N^{ω}-nitro-L-arginine methyl ester (L-NAME), a non-specific inhibitor of NOS, was added to the bath 30 min before performing the second frequency–response curve. The vasodilator response to the NO donor, diethylamine NONOate, (DEA-NO, 0.1 nmol·L^{-1} to 0.1 mmol·L^{-1}), was determined in NA-precontracted arteries from both experimental groups.

NA and ATP release

Endothelium-denuded segments of rat mesenteric arteries from O-CR and O-DR animals were pre-incubated for 30 min in 5 mL of KHS at 37°C and continuously gassed with a 95% O_2 to 5% CO_2 mixture (stabilization period). This was followed by two washout periods of 10 min in a bath of 0.4 mL KHS. Then the medium was collected to measure basal neurotransmitter release. Next, the organ bath was refilled and cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz were applied at 1 min intervals. Afterwards, the medium was collected to measure the EFS-induced release. Mesenteric segments were weighed in order to normalize the results. NA and ATP release were measured using Noradrenaline Research EIA (Labor Diagnostica Nord, Gmbh and Co., KG, Nordhon, Germany) or an ATP colorimetric/fluorometric assay kit (Abcam, Cambridge, UK). The assays were performed following the manufacturer's instructions. Results are expressed as ng NA·mL^{-1} mg^{-1} tissue, or nmol ATP·mL^{-1} mg^{-1} tissue.

NO release

NO release was measured using fluorescence emitted by the fluorescent probe 4,5-diaminofluorescein (DAF-2) as previously described (Blanco-Rivero *et al.*, 2011a). Endothelium-denuded mesenteric arteries from O-CR and O-DR rats were subjected to a 60 min equilibration period in HEPES buffer [in mmol·L^{-1}: NaCl 119; HEPES 20; CaCl2 1.2; KCl 4.6; $MgSO_4$ 1; KH_2PO_4 0.4; $NaHCO_3$ 5; glucose 5.5; Na_2HPO_4 0.15; (pH 7.4)] at 37°C. Arteries were incubated with 2 μmol·L^{-1} DAF-2 for 30 min. The medium was collected to measure basal NO release. Once the organ bath was refilled, cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz were applied at 1 min intervals. Afterwards, the medium was collected to measure EFS-induced NO release. The fluorescence of the medium was measured at room temperature (RT) using a spectrofluorometer (LS50 PerkinElmer Instruments, FL WINLAB Software, Waltham, MA, USA) with excitation wavelength set at 492 nm and emission wavelength at 515 nm.

The EFS-induced NO release was calculated by subtracting basal NO release from that evoked by EFS. Also, blank samples were collected in the same way from segment-free medium in order to subtract background emission. Some assays were performed in the presence of 0.1 mmol·L^{-1} TTX or 1 mmol·L^{-1} 1400 W, the specific iNOS inhibitor. The amount of NO released was expressed as arbitrary units·mg^{-1} tissue.

Superoxide anion production

Superoxide anion levels were measured using lucigenin chemiluminescence (Blanco-Rivero *et al.*, 2011b). Endothelium-denuded mesenteric segments from O-CR and O-DR animals were rinsed in KHS for 30 min, equilibrated for 30 min in HEPES buffer at 37°C, transferred to test tubes that contained 1 mL HEPES buffer (pH 7.4) with lucigenin (5 μmol·L^{-1}) and then kept at 37°C. The luminometer was set to report arbitrary units of emitted light; repeated measurements were collected for 5 min at 10 s intervals and averaged. 4,5-Dihydroxy-1,3-benzene-disulfonic acid 'Tiron' (10 mmol·L^{-1}), a cell-permeant, non-enzymatic superoxide anion scavenger, was added to quench the superoxide anion-dependent chemiluminescence. Also, blank samples were collected in the same way without mesenteric segments to subtract background emission.

Immunofluorescence staining of nerve fibres

SMA was immediately placed in cold PBS (in g·L^{-1}): NaCl 8, Na_2HPO_4 1.15, KCl 0.2, KH_2PO_4 0.2 (pH 7.2). The whole vessel was fixed (4% paraformaldehyde in PBS, 50 min, RT). After three 10 min PBS washing cycles, non-specific binding was blocked by incubating the samples for 1 h in 5% bovine albumin PBS + 0.3% Tween 20 (PBS-T). The vessels were cut into segments and incubated with primary antibodies: rabbit polyclonal anti-dopamine β-hydroxylase (Sigma 1:200) or rabbit polyclonal anti-neuronal NOS (nNOS; Abcam 1:100) diluted in 2% bovine albumin PBS-T. Thereafter, tissues were stained with the nuclear dye DAPI (1:500 dilution, 15 min, RT) and, after two PBS-T washing cycles, incubation with Alexa 647 anti-rabbit fluorescent secondary antibodies (1:200 dilution, 1 h, RT) was carried out. Negative controls were performed by omitting primary antibodies. After four PBS-T washing cycles, tissue preparations were mounted in a single well filled with antifading agent (Citifluor AF-2, Citifluor Ltd).

Preparations were visualized with a Leica SP5 laser scanning confocal microscopy (LSCM) system (Leica Microsystems, Wetzlar, Germany) fitted with an inverted microscope (×40 oil immersion lens). Stacks of 2 μm thick serial optical images were captured from the entire adventitial layer, which was identified by the shape and orientation of the nuclei (Arribas *et al.*, 1997). Image acquisition was always performed under the same laser power, brightness and contrast conditions.

Two to three different regions were scanned along each mesenteric artery, and the resulting images were

reconstructed separately with ImageJ 1.48c software (National Institutes of Health) to generate extended focus images. After obtaining the 0 value for the background, the area fraction parameter was set to determine the areas occupied by fibres defined as percentage of pixels with non-zero. Data are presented as percentage of area occupied by nerve fibres.

nNOs and phospho-nNOS (P-nNOS) expression

Western blot analysis of nNOS and P-nNOS expression was performed in endothelium-denuded mesenteric segments from O-CR and O-DR rats as described previously (Blanco-Rivero *et al*., 2011a,b). For these experiments, we used mouse monoclonal nNOS antibody (1:1000, Transduction Laboratories), rabbit polyclonal P-nNOS antibody (1:2000, Abcam) and monoclonal anti-β-actin-peroxidase antibody (1:50 000, Sigma-Aldrich, Madrid, Spain). Rat brain homogenates were used as positive control.

Drugs used

L-Noradrenaline hydrochloride, ACh chloride, diethylamine NONOate diethylammonium salt, CGRP (8–37), TTX, L-NAME hydrochloride, 1400 W, phentolamine, suramin sodium salt, lucigenin, tiron and DAF-2 (Sigma-Aldrich) were used. Stock solutions (10 mmol·L^{-1}) of drugs were made in distilled water, except for NA, which was dissolved in a NaCl (0.9%) ascorbic acid (0.01% w v^{-1}) solution. These solutions were kept at –20°C and appropriate dilutions were made in KHS on the day of the experiment.

Data analysis

The responses elicited by EFS and NA were expressed as a percentage of the initial contraction elicited by 75 mmol·L^{-1} KCl for comparison between O-CR and O-DR rats. The relaxation induced by ACh or DEA-NO was expressed as a percentage of the initial contraction elicited by NA (control: 10.36 ± 0.85 mg; O-DR: 10.81 ± 0.55 mg; $P > 0.05$). For concentration–response curves, non-linear regression was performed. Results are given as mean ± SEM. Statistical analysis was carried out by comparing the curve obtained in the presence of different substances with the previous or control curve by means of repeated measure ANOVA followed by the Bonferroni *post hoc* test. Some results are expressed as differences in AUC (dAUC). AUCs were calculated from the individual frequency–response plots. For dAUC, NO, NA and ATP release experiments, the statistical analysis was performed using one-way ANOVA followed by Newman–Keuls *post hoc* test. Glucose tolerance, insulin sensitivity, arterial blood pressure, heart rate, results of general characteristics and immunofluorescence staining were analysed using *t*-test. $P < 0.05$ was considered significant.

Results

Dams injected with streptozotocin had severe hyperglycaemia on gestational days 14 (control 829 ± 31 vs. diabetic 4572 ± 337 mg·L^{-1}, *t*-test: $P < 0.05$) and 21 (control 872 ± 26 vs. diabetic 4806 ± 394 mg·L^{-1}, *t*-test: $P < 0.05$) compared with control dams. Blood glucose levels were similar in O-CR an O-DR in 3-month-old (O-CR 764 ± 37 vs. O-DR 743 ± 28 mg·L^{-1}, *t*-test: $P > 0.05$) and 6-month-old animals (O-CR 788 ± 62 vs. O-DR 792 ± 78 mg·L^{-1}, *t*-test: $P > 0.05$).

The oral glucose tolerance test revealed that blood glucose levels were higher in O-DR at 30 min compared with O-CR (results not shown) and remained increased until 120 min (O-CR, 1133 ± 97 vs. O-DR, 1568 ± 34 mg·L^{-1}, *t*-test: $P < 0.05$). Results from the insulin sensitivity test demonstrated significant insulin resistance in the O-DR as they presented a higher blood glucose level from 30 to 60 min after an insulin injection (blood glucose 60 min after the insulin injection: O-CR, 230 ± 15 vs. O-DR, 612 ± 97 mg·L^{-1}; *t*-test: $P < 0.05$).

O-DR had a higher blood pressure than O-CR (mean arterial pressure: O-CR: 119 ± 6.5 vs. O-DR: 137 ± 0.5 mmHg; *t*-test: $P < 0.05$). The heart rate was similar in both the O-DR and the O-CR (O-CR: 392 ± 21 vs. O-DR: 397 ± 32 bpm; *t*-test: $P > 0.05$).

Body weight was decreased in the O-DR group (Table 1). Serum insulin, total cholesterol, HDL and triglycerides were similar in both experimental groups (Table 1).

Table 1

Body weight and serum biochemical parameters

	O-CR	O-DR
Body weight (mg)	438.8 ± 17.16	381.3 ± 8.25*
Total cholesterol (mg·mL^{-1})	0.80 ± 0.07	0.73 ± 0.04
HDL (mg·mL^{-1})	0.52 ± 0.04	0.48 ± 0.02
Triglycerides (mg·mL^{-1})	1.32 ± 0.12	1.27 ± 0.17
Insulin (ng·mL^{-1})	5.1 ± 0.3	4.8 ± 0.1

At 6 months, body weight was measured, total cholesterol, HDL cholesterol, triglycerides and insulin in O-CR and O-DR animals. Data shown are means ± SEM. *$P < 0.05$ versus O-CR. $n = 8$ animals per group.

Vasomotor response to KCl

In endothelium-intact mesenteric segments, the vasoconstrictor response to 75 mmol·L^{-1} KCl was similar in both experimental groups (O-CR: 13.8 ± 1.1 mN; O-DR: 15.53 ± 1.18 mN; $P > 0.05$). Endothelium removal did not alter KCl-induced vasoconstriction (O-CR: 15.21 ± 1.5 mN; O-DR: 14.59 ± 10.9 mN; $P > 0.05$).

Vasodilator response to ACh

ACh-induced cumulative concentration- and endothelium-dependent relaxations in NA-contracted arteries (O-CR: 10.26 ± 0.84 mN; O-DR: 10.71 ± 0.55 mN; $P > 0.05$) from both the O-CR and O-DR groups. However, the exposure to maternal diabetes decreased this vasodilator response as compared with the response in the O-CR group (Figure 1A).

Vascular responses to EFS

The application of EFS induced a frequency-dependent contractile response in endothelium-intact mesenteric segments from both the O-CR and O-DR groups. This vasoconstriction

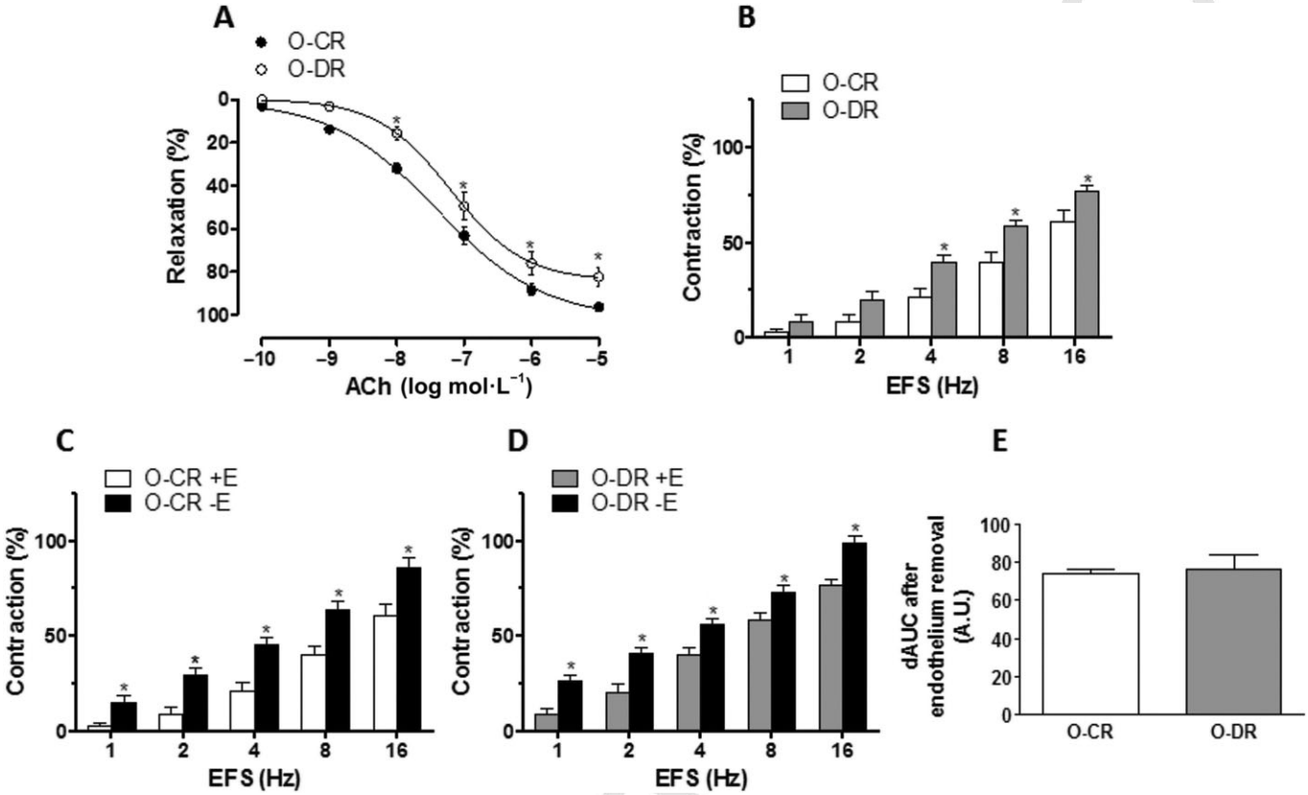

Figure 1

(A) ACh-induced vasodilatation in endothelium-intact mesenteric segments from O-CR and O-DR rats. Results (mean ± SEM) are expressed as a percentage of the previous tone elicited by exogenous NA. ANOVA *$P < 0.05$ O-CR versus O-DR. $n = 12$ animals in each group. (B) EFS-induced vasoconstriction in endothelium-intact mesenteric segments from O-CR and O-DR rats. Results (mean ± SEM) are expressed as a percentage of the initial contraction elicited by KCl. *$P < 0.05$ versus O-CR animals at each frequency (Bonferroni test). $n = 12$ animals per group. Effect of endothelium removal on the vasoconstrictor response to EFS in mesenteric segments from O-CR (C) and O-DR (D) animals. Results (mean ± SEM) are expressed as a percentage of the initial contraction elicited by KCl. ANOVA *$P < 0.05$ endothelium-intact versus endothelium-denuded arteries. $n = 12$ animals per group. (E) dAUC in the absence or presence of endothelium.

Table 2

Effect of pre-incubation with TTX (0.1 μmol·L^{-1}) on the frequency–contraction curves performed in mesenteric segments from O-CR and O-DR

	1 Hz	2 Hz	4 Hz	8 Hz	16 Hz
O-CR	8.74 ± 2.9	25.45 ± 4.1	41.44 ± 5.7	58.49 ± 6.9	81.92 ± 8.5
TTX	0	0	0	0.4 ± 0.06	0.7 ± 0.1
O-DR	21.61 ± 6.8	37.92 ± 6.4	35.53 ± 6.9	74.05 ± 7.1	100.49 ± 8.5
TTX	0	0	0	0.5 ± 0.04	0.9 ± 0.02

Results (means ± SEM) are expressed as percentages of the response elicited by 75 mM KCl; 0, no contraction was detected. $n = 7$ animals for each group.

was greater in segments from O-DR rats compared with O-CR animals (Figure 1B). Endothelium removal increased EFS-induced contractile response similarly in segments from all experimental groups (Figure 1C and D). EFS-induced contractions were practically abolished in segments from all experimental groups by the neurotoxin TTX (0.1 mmol·L^{-1}), indicating the neuronal origin of the factors inducing this response (Table 2).

Participation of the sympathetic component of mesenteric vascular neurons

Pre-incubation with the α-adrenoceptor antagonist phentolamine (1 μmol·L^{-1}) decreased the vasoconstrictor response induced by EFS in endothelium-denuded segments from both experimental groups (Figure 2A and B). This decrease was greater in mesenteric segments from O-DR animals (Figure 2C). NA-induced vasoconstriction was similar in both experimental groups (Figure 3A). EFS-induced NA release was higher in mesenteric segments from the O-DR group than in segments from O-CR animals (Figure 3B). A phentolamine-resistant contractile response remained, which was greater in mesenteric segments from O-DR animals (Figure 2D). Pre-incubation with phentolamine plus 0.1 mmol·L^{-1} suramin, a non-specific P2 purine receptor antagonist, decreased the EFS-induced contraction only in segments from the O-DR group (Figure 2A and B). In line with this, EFS-induced ATP release was increased in segments from O-DR animals (Figure 3C).

LSCM allowed visualization of the sympathetic nerve network in the adventitial layer of mesenteric arteries through reconstruction of stacks of images with dopamine β-hydroxylase immunoreactivity (Figure 4A and B). Quantification of nerve fibre density confirmed that there were no differences between either experimental groups (Figure 4C).

Participation of the sensory component in vascular responses to EFS

Pre-incubation with the CGRP receptor antagonist CGRP (8–37) (0.5 μmol·L^{-1}) did not alter the EFS-induced contraction in any experimental group (Figure 5).

Participation of the nitrergic component in vascular responses to EFS

Pre-incubation with non-specific NOS inhibitor L-NAME (0.1 mmol·L^{-1}) significantly increased the EFS–contractile response in endothelium-denuded segments from both experimental groups (Figure 6A and B). This increase was greater in segments from O-DR animals (Figure 6C). EFS induced NO release in segments from both groups. This release was higher in O-DR mesenteric segments (Figure 7A). TTX practically abolished EFS-induced NO release, whereas 1400 W did not modify it in either experimental group (Figure 7A). The expression of nNOS was not modified, whereas P-nNOS expression was increased in homogenates from O-DR arteries compared with that in O-CR segment homogenates (Figure 7B). Maternal gestational diabetes did not alter either the vasodilator response to DEA-NO (NA pre-contraction: control: 10.33 ± 0.74 mN; O-DR: 10.46 ± 0.65 mN; $P > 0.05$) or the superoxide anion release in the offspring (Figure 7C and D).

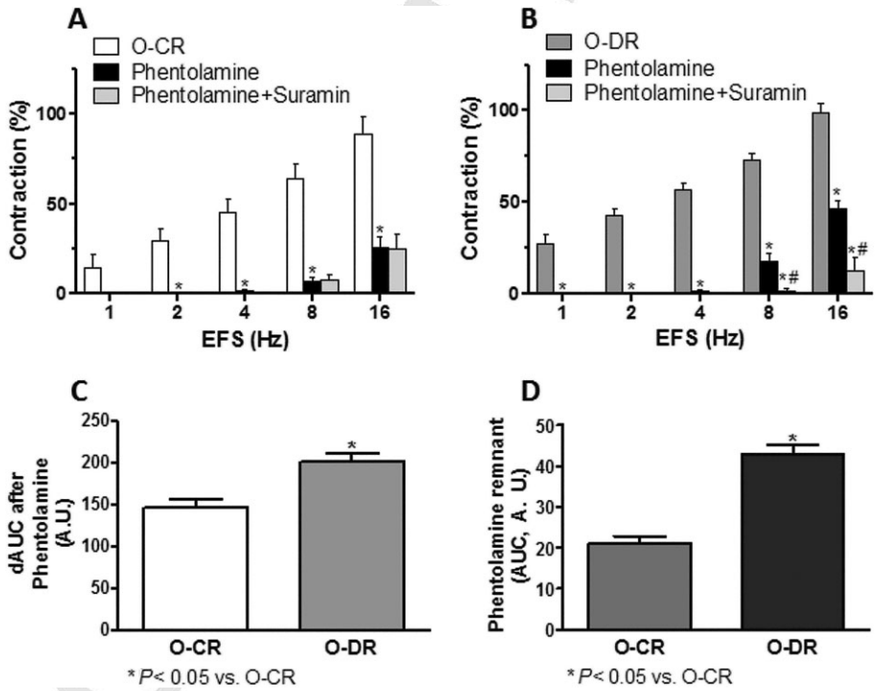

Figure 2

Effect of pre-incubation with 1 μmol·L^{-1} phentolamine or phentolamine plus 0.1 mmol·L^{-1} suramin on the vasoconstrictor response induced by EFS in endothelium-denuded mesenteric segments from O-CR (A) and O-DR animals (B). Results (mean ± SEM) are expressed as a percentage of the initial contraction elicited by KCl. ANOVA $P < 0.05$ versus conditions without phentolamine or suramin in both experimental groups. *$P < 0.05$ versus conditions without phentolamine at each frequency (Bonferroni test). #$P < 0.05$ conditions phentolamine plus suramin versus conditions without suramin at each frequency (Bonferroni test). $n = 8$ animals each group. (C) dAUC in the absence or presence of 1 μmol·L^{-1} phentolamine. dAUC values are expressed as arbitrary units. (D) Representation of the vasoconstriction remaining after pre-incubation with 1 μmol·L^{-1} phentolamine, expressed as AUC (in arbitrary units).

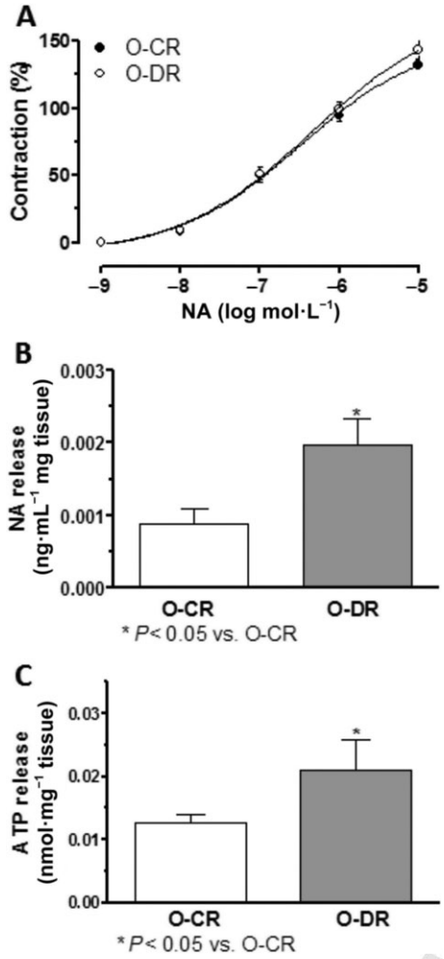

Figure 3

(A) Vasoconstrictor response to NA in segments from O-CR and O-DR. Results (mean ± SEM) are expressed as a percentage of the initial contraction elicited by KCl. n = 8 animals each group. EFS-induced NA (B) and ATP (C) release in mesenteric segments from O-CR and O-DR animals. Results (mean ± SEM) are expressed as ng NA·mL^{-1} mg^{-1} tissue or nmol ATP·mL^{-1} mg^{-1} tissue. n = 8 animals per group.

LSCM allowed visualization of the nitrergic nerve network in the adventitial layer of mesenteric arteries through reconstruction of stacks of images with nNOS immunoreactivity (Figure 8A and B). Quantification of nerve fibre density confirmed the absence of differences between both experimental groups (Figure 8C).

Discussion

This study provides the first evidence that maternal diabetes alters the participation of different kinds of perivascular mesenteric neurons in adult offspring. The results presented here demonstrate that *in utero* exposure to maternal hyperglycaemia increases EFS-induced vasoconstriction in adulthood. This increase is endothelium independent, and is the net effect of an increased participation of sympathetic neurons, through increased release of NA and ATP, and an augmented activity of nitrergic neurons, associated with increased NO production.

The body weight of rats in the O-DR group was significantly lower compared with those in the O-CR group. This result agrees with previous reports showing that maternal diabetes induced by STZ produces a decrease in offspring weight (Grill *et al.*, 1991; Holemans *et al.*, 1999; Porto *et al.*, 2010; Ramos-Alves *et al.*, 2012a) that has been associated with reduced protein synthesis (Canavan and Goldspink, 1988). However, several studies have found no changes or even increases in the body weight of the offspring of rats subjected to this experimental procedure (Rocha *et al.*, 2005; Nehiri *et al.*, 2008). This apparent contradiction may be due to differences in the severity of maternal hyperglycaemia during pregnancy, as previously suggested by Segar *et al.* (2009).

Intrauterine exposure to maternal hyperglycaemia is a significant risk factor for the development of metabolic and cardiovascular disorders in the offspring. In the current study, 6-month-old male offspring of diabetic mothers exhibited glucose intolerance, insulin resistance and an elevated blood pressure, as we (Ramos-Alves *et al.*, 2012a,b) and others have reported previously (Simeoni and Barker, 2009). Glucose and insulin levels and lipid profile were similar in both experimental groups, in agreement with Blondeau *et al.* (2011). The mechanisms of hyperglycaemia-programmed hypertension are complex and involve renal, neural and vascular factors (Rocha *et al.*, 2005; Wichi *et al.*, 2005; Nehiri *et al.*, 2008; Segar *et al.*, 2009; Chen *et al.*, 2010). The analysis of the vasoconstrictor response induced by EFS in endothelium-intact segments showed a frequency-dependent contraction in segments from both experimental groups. This vasoconstriction was greater in mesenteric segments from O-DR than O-CR animals. This increase could not be attributed to changes in the intrinsic contractile machinery as a similar vasoconstrictor response to KCl was obtained in both experimental groups. The endothelium has been reported to affect the response to several substances including neurotransmitters such as NA (Vanhoutte and Houston, 1985; Li and Duckles, 1992). Because we observed that the relaxation to ACh was impaired in O-DR compared with O-CR rats, as we and other groups had previously reported (Holemans *et al.*, 1999; Rocha *et al.*, 2005; Segar *et al.*, 2009; Ramos-Alves *et al.*, 2012a), we expected differences in the influence of endothelium on the vasoconstrictor response to EFS in segments from the two experimental groups. However, endothelium removal increased this vasoconstriction to the same extent in both experimental groups. These results indicate that endothelial dysfunction does not influence the EFS-induced response in diabetic offspring, similar to reports on this artery in rats fed a high fat diet (Sastre *et al.*, 2015). One possible explanation could be that the vasoconstrictor response to α-adrenoceptor activation has been reported as maintained in the absence of endothelium (Xavier *et al.*, 2004a) independently of the modifications of the vasodilator response to ACh. In endothelium-denuded segments, the EFS-induced vasoconstriction was almost abolished in the presence of the neurotoxin TTX, indicating a neuronal origin for this

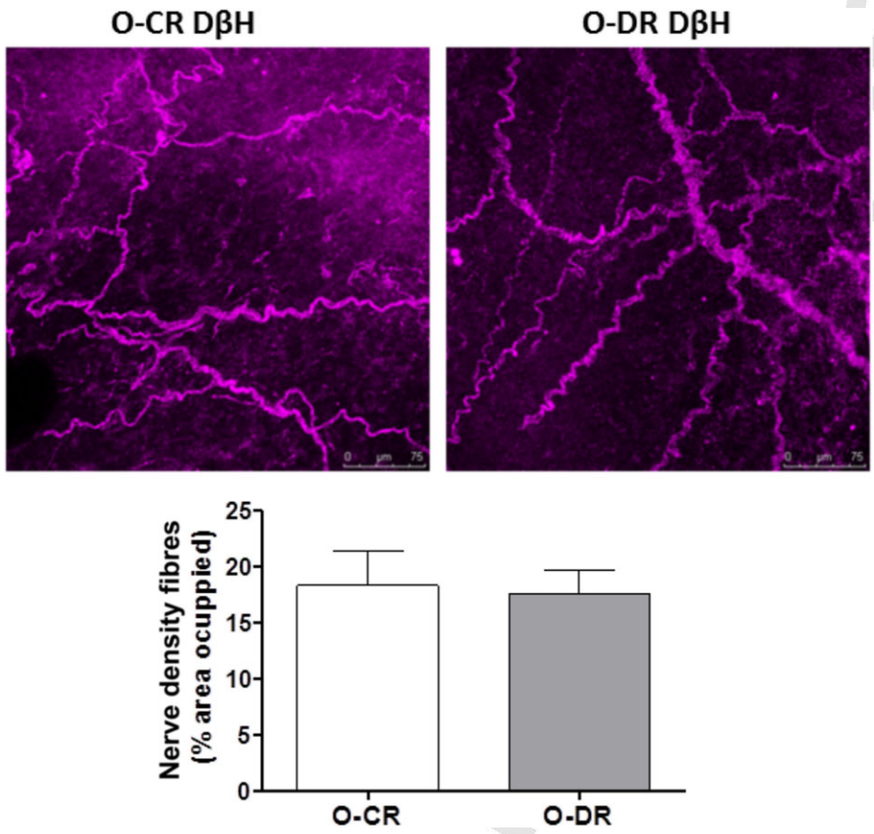

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Figure 4

Dopamine β-hydroxylase immunoreactivity in the adventitia of mesenteric arteries from O-CR (A) and O-DR (B) animals. Tissues were stained with primary monoclonal dopamine β-hydroxylase (DβH) antibody and a species-specific secondary Alexa 647 antibody. Bar = 75 µm. All images are reconstructions from 10 serial optical sections obtained by LSCM. (C) Percentage of area occupied by sympathetic nerve fibres. $n = 6$ animals per group. (For a better visualization, brightness and contrast were modified equally in both experimental groups using ImageJ software).

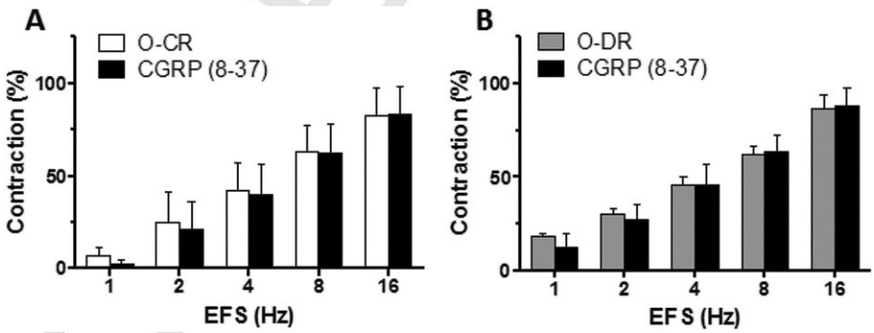

Figure 5

Effect of pre-incubation with 0.5 µmol·L^{-1} CGRP (8–37) on the vasoconstrictor response induced by EFS in mesenteric segments from O-CR (A) and O-DR (B) animals. Results (mean ± SEM) are expressed as a percentage of the previous contraction elicited by KCl. $n = 8$ animals each group.

response. The remaining contractile response to high-frequency stimulation could be explained by a direct action potential produced in smooth muscle cells elicited by EFS. For that reason, we performed the following experiments in endothelium-denuded mesenteric segments.

Sympathetic function is essential for blood pressure regulation, and sympathetic hyperactivity has an important role in the development of hypertension (Lohmeier, 2001). Sympathetic neurons mainly release NA and ATP when electrically stimulated. The activity of these neurons is altered in

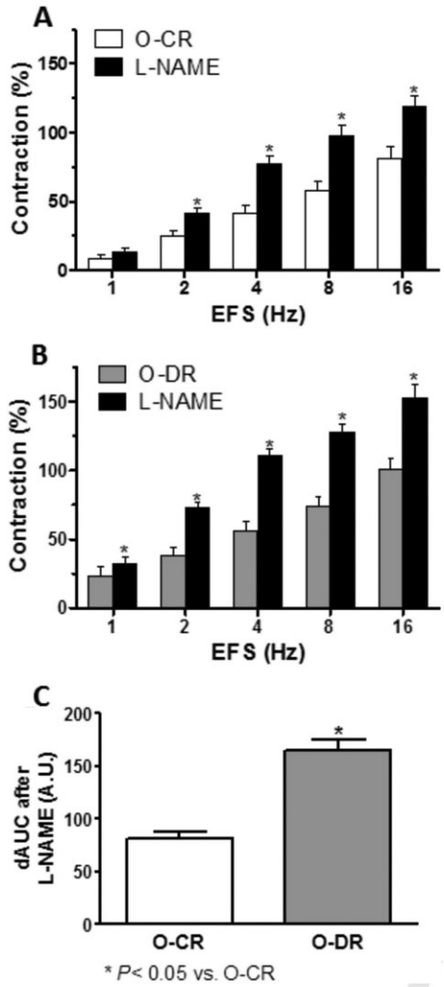

Figure 6

Effect of pre-incubation with 0.1 mmol·L^{-1} L-NAME on the vasoconstrictor response induced by EFS in mesenteric segments from O-CR (A) and O-DR (B) rats. Results are expressed as a percentage of the previous contraction elicited by KCl. ANOVA $P < 0.05$ versus conditions without phentolamine or suramin in both experimental groups. *$P <$ 0.05 versus conditions without L-NAME for each frequency (Bonferroni test). $n = 8$ animals each group. (C) dAUC in the absence or presence of 0.1 mmol·L^{-1} L-NAME. dAUC values are expressed as arbitrary units. *$P < 0.05$.

different physiological and pathological situations (Sastre *et al.*, 2010; 2012a). Increased sympathetic activity has been reported in O-DR (Young and Morrison, 1998; Iellamo *et al.*, 2006; de Almeida Chaves Rodrigues *et al.*, 2013). This effect could be associated with an increased NA release and/or vasoconstrictor response to NA. To the best of our knowledge, although increased NA release in different tissues has been suggested (Morriss, 1984; Iellamo *et al.*, 2006), as well as increases or no modifications in NA vasoconstriction (Holemans *et al.*, 1999; Ramos-Alves *et al.*, 2012b), an integrated study of both mechanisms has not yet been performed in arteries. Thus, our next objective was to determine possible differences in the function of sympathetic neurons between the O-CR and O-DR experimental groups. The fact that the α-adrenoceptor antagonist phentolamine significantly diminished the vasoconstrictor response to EFS in mesenteric segments from both experimental groups confirms that this response would be mediated mainly by the release of NA from sympathetic nerve terminals. Moreover, our study showed that phentolamine produced a more marked decrease in EFS vasoconstriction in segments from OD-R rats than O-CR animals, confirming an increased involvement of sympathetic neurons in this experimental group. This different participation can be produced by modifications in NA vasoconstrictor response and/or release. Exogenous NA-induced vasoconstriction was similar in both experimental groups, like the vasoconstriction described in endothelium-denuded mesenteric resistance arteries (Ramos-Alves *et al.*, 2012b), whereas EFS-induced NA release was higher in segments from O-DR animals, confirming that the increased adrenergic activity in O-DR is associated with increased NA release.

It should also be mentioned that both groups showed a substantial phentolamine-resistant contractile response. This remaining vasoconstriction was higher in segments from O-DR animals. We have previously observed that ATP released from sympathetic nerves caused vasoconstriction in this vascular bed (Blanco-Rivero *et al.*, 2011a; 2013a,b). Based on this information, we analysed EFS-induced contraction after simultaneous pre-incubation with phentolamine plus the non-specific P2 purine receptor antagonist suramin. In these conditions, the contractile response to EFS was reduced only in segments from the O-DR group, indicating that ATP contributes to this response. In line with these results, EFS-induced ATP release was higher in mesenteric segments from O-DR than O-CR rats, as previously described (Rummery *et al.*, 2007; Sousa *et al.*, 2014), suggesting an increased contribution of ATP in neurovascular transmission in hypertension. Altogether, these observations indicate that the increased participation of sympathetic neurons observed in mesenteric segments from O-DR animals is due to an increase in NA and ATP release from nerve terminals. Because this increased release of sympathetic neurotransmitters could be due to an increase in nerve fibre density, our next objective was to determine possible differences in the sympathetic nerve density. Results obtained by immunofluorescence staining for dopamine β-hydroxylase showed no differences between either experimental group, ruling out an increase in sympathetic nerve density and suggest that the augmented release of NA and ATP could be related to an increase in enzymatic activity involved in the synthesis of both neurotransmitters. However, neurotransmitter release by sympathetic neurons is mediated by several subtypes of adrenoceptor (Sanchez-Merino *et al.*, 1990; Arribas *et al.*, 1991; Enero *et al.*, 1997; Kanagy, 2005), so some type of presynaptic dysfunction cannot be ruled out.

We have previously reported that CGRP released from sensory neurons does not participate in the vasoconstrictor response to EFS (Blanco-Rivero *et al.*, 2011a,c), although the level of CGRP is increased in several pathological situations, such as hypertension and diabetes (Blanco-Rivero *et al.*, 2011c; del Campo *et al.*, 2011; Sastre *et al.*, 2012a). Pre-incubation with the CGRP receptor antagonist CGRP (8–37) did not modify EFS-induced vasoconstriction in either O-CR or O-DR mesenteric segments. This observation led us to

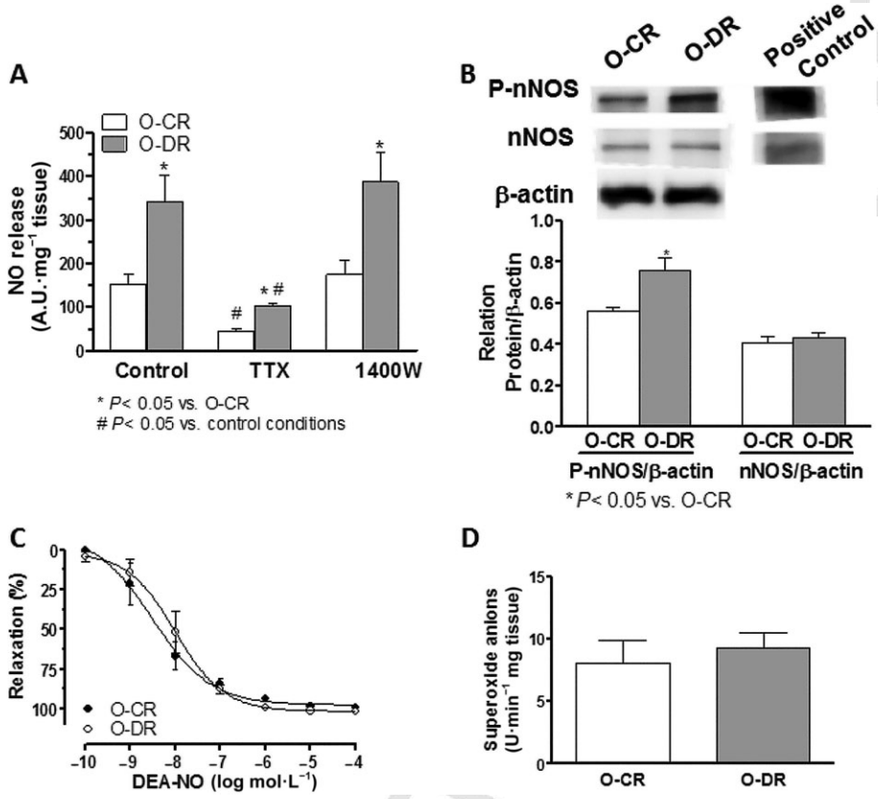

Figure 7

(A) EFS-induced NO release in segments from O-CR and O-DR rats. Results (mean ± SEM) are expressed as arbitrary (A.U.)·mg^{-1} tissue; n = 8 animals per group. (B) Effect of exposure to maternal hyperglycaemia on nNOS and P-nNOS expression. The blot is representative of eight separate segments from each group. Rat brain homogenates were used as a positive control. Lower panel shows relationship between P-nNOS or nNOS expression and β-actin. Results (mean ± SEM) are expressed as ratio of the signal obtained for each protein and the signal obtained for β-actin. (C) Vasodilator response to NO donor DEA-NO in segments from O-CR and O-DR rats. Results (mean ± SEM) are expressed as a percentage of the previous tone elicited by exogenous NA. n = 8 animals each group. (D) Superoxide anion release in mesenteric segments from O-CR and O-DR rats. Results (mean ± SEM) are expressed as chemiluminescence U·min^{-1} mg tissue. n = 8 animals each group.

conclude that maternal diabetes did not alter the activity of sensory neurons in this vascular bed in adulthood.

A decrease in endothelial NO release has been reported to participate in the development of hypertension (Wu *et al.*, 1997; Bernatova, 2014). Similar results have been observed in rat aorta and mesenteric resistance arteries from offspring of diabetic rats (Holemans *et al.*, 1999; Cavanal Mde *et al.*, 2007; Ramos-Alves *et al.*, 2012a). However, there are no data studying the possible role of neuronal NO in this model. The fact that previous studies have reported increases in endothelial NO release due to ATP-induced activation of P2X4 and P2Y receptors and augmented NOS activity due to endothelial α_2 adrenoceptor activation by NA in rat mesenteric arteries (Boric *et al.*, 1999; Buvinic *et al.*, 2002; Codocedo *et al.*, 2013), as well as our earlier report of increased neuronal NO in both diabetes and hypertension (Ferrer *et al.*, 2000; 2003; Marín *et al.*, 2000; del Campo *et al.*, 2011), make it necessary to evaluate whether a hyperglycaemic environment *intra utero* could also affect the synthesis of NO from nitrergic neurons. In our experimental conditions, the involvement of neuronal NO in the EFS-induced response was demonstrated by the fact that pre-incubation with the non-specific NOS inhibitor L-NAME increased the response to EFS in segments from both experimental groups. The greater effect of L-NAME observed in segments from O-DR rats suggests an increased role for neuronal NO in this experimental group, possibly related to increases in nitric NO and/or increases in smooth muscle sensitivity to NO.

In our experimental conditions, we observed an increased EFS-induced NO release in mesenteric segments from O-DR animals. The fact that pre-incubation with TTX abolished EFS-induced NO release in segments from both groups of rats, and that pre-incubation with the specific iNOS inhibitor 1400 W did not alter NO release confirms the neural origin of NO and rules out the involvement of inducible NOS. We have previously demonstrated that NO released from nerve endings in this vascular bed is synthesized through nNOS (Blanco-Rivero *et al.*, 2011a,b; 2013a,b). The increase in neuronal NO could be due to an increase in nitrergic nerve fibre density or an increase in the enzymatic activity of nNOS. Thus, our next objective was to determine possible differences in nitrergic nerve density. Immunofluorescence staining for nNOS showed no differences between experimental groups, suggesting that the differences in NO release could be

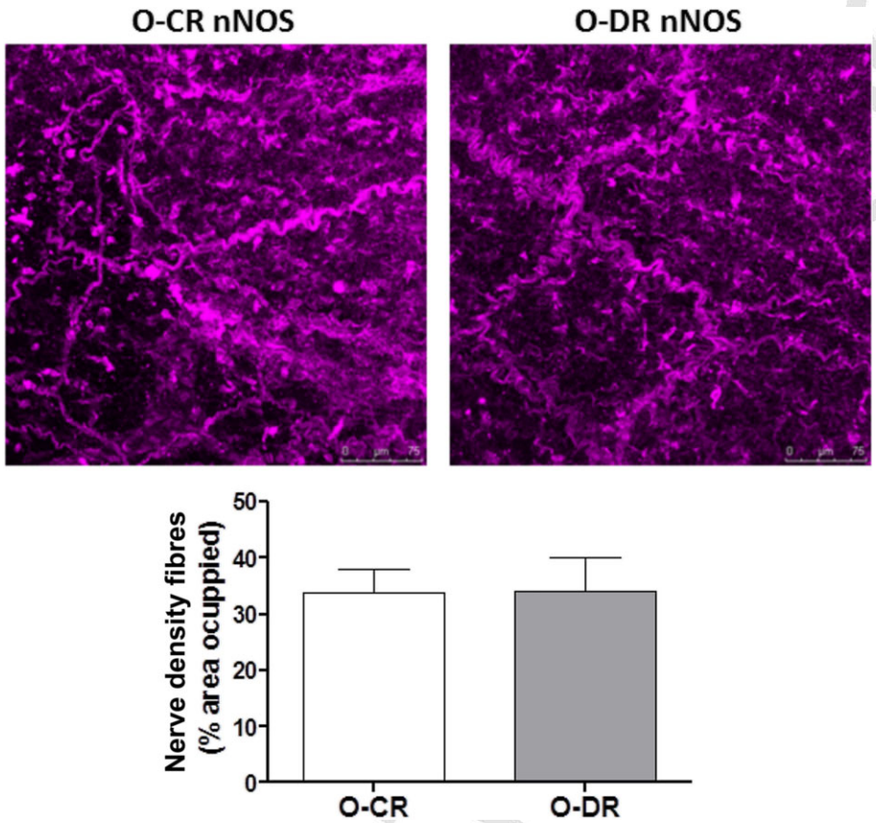

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Figure 8

nNOS immunoreactivity in the adventitia of mesenteric arteries from O-CR (A) and O-DR (B) animals. Tissues were stained with primary polyclonal nNOS antibody and a species-specific secondary Alexa 647 antibody. Bar = 75 µm. All images are reconstructions from 10 serial optical sections obtained by LSCM. (C) Percentage of area occupied by nitrergic nerve fibres. *n* = 6 animals per group.

due to modifications in the expression and/or activation of nNOS. We found that nNOS protein expression was similar in both experimental groups, whereas the active form P-nNOS was increased in O-DR rats, indicating that increased nNOS activity is responsible for the increase in NO release observed in O-DR mesenteric arteries. These results contrast with observations in endothelium-intact arteries (Holemans *et al.*, 1999; Rocha *et al.*, 2005; Segar *et al.*, 2009; Porto *et al.*, 2010), where the NO release from eNOS was decreased. Similar differences in this vascular bed have been demonstrated previously in several pathological situations (Wu *et al.*, 1997; Ferrer *et al.*, 2000; 2003; Favero *et al.*, 2014).

Several observations indicate that diabetic pregnant rats and their offspring are exposed to increased oxidative stress induced by the inadequate production of free radical scavengers (Horal *et al.*, 2004; Li *et al.*, 2005; Katkhuda *et al.*, 2012). Thus, the involvement of reactive oxidative species in the vascular response to NO cannot be ruled out as these species could alter the metabolism and consequently affect neuronal NO bioavailability. Superoxide anion production was similar in both experimental groups, in contrast to findings from previous studies (Horal *et al.*, 2004; Li *et al.*, 2005; Katkhuda *et al.*, 2012). These differences can be attributed to the different tissues analysed as well as to the method of inducing maternal diabetes. As was expected from the earlier results, the vasodilator response to the NO donor DEA-NO was similar in segments from both experimental groups, and similar to responses in aorta and mesenteric resistance arteries (Holemans *et al.*, 1999; Katkhuda *et al.*, 2012; Ramos-Alves *et al.*, 2012a). Altogether, these results confirm that the increased activity of the nitrergic neurons is due to increased neuronal NO release and not to changes in the vasodilator response and/or metabolism of neuronal NO.

A reciprocal interaction between sympathetic and nitrergic neurons has been demonstrated in several vascular beds (Lee, 2002; Hatanaka *et al.*, 2006; Koyama *et al.*, 2010). However, in SMA, we have demonstrated an increase in nitrergic activity with no change (Xavier *et al.*, 2004b), a decrease (Sastre *et al.*, 2012a,b) or increase (Ferrer *et al.*, 2000; 2003; Marín *et al.*, 2000; del Campo *et al.*, 2011) in adrenergic activity, as in the current study. These observations indicate that modifications in the activity of adrenergic and nitrergic neurons in OD-R are independent and lead us to consider them as primary changes.

Based on previous studies, various hypotheses can be raised about the mechanism/s involved in these effects. During pregnancy, maternal high blood glucose levels stimulate the fetal pancreas to produce high insulin levels (Dabelea, 2007). Hyperinsulinaemia may cause sympathetic overactivation due its effects on the CNS (Scherrer and

Sartori, 1997). In the offspring of diabetic rats, a decrease in the mean area of neuronal nuclei and neuronal cytoplasm has been demonstrated in the ventromedial hypothalamic nucleus, paraventricular hypothalamic nucleus and arcuate nucleus (Plagemann *et al.*, 1999). These changes were accompanied by an increased number of neurons expressing tyrosine hydroxylase in the arcuate nucleus. It has been proposed that this increase in tyrosine hydroxylase-expressing neurons might activate and overstimulate the differentiation of hypothalamic catecholaminergic systems due to hyperinsulinism. Previous reports have demonstrated that hyperinsulinaemia may also increase the phosphorylation of nNOS (Chiang *et al.*, 2009; Fioramonti *et al.*, 2010; Hinchee-Rodriguez *et al.*, 2013) and thereby increase neuronal NO release in the offspring of diabetic dams. Additionally, fetal leptin levels are also raised by maternal hyperglycaemia, which may contribute to central leptin resistance and hypothalamic dysfunction (Steculorum and Bouret, 2011) in the offspring of diabetic rats. Thus, it is possible to postulate that hormonal changes produced by hyperglycaemia during fetal life induce changes in neuronal activity/development that persist to some extent into adult life, as observed in the current study.

In conclusion, maternal diabetes increases the contractile response to EFS in SMA of their adult offspring. This effect is endothelium-independent and is the net result of increased levels of sympathetic vasoconstrictors NA and ATP along with an augmented release of neuronal NO, whereas the activity of sensory neurons is unaltered. This mechanism could be involved in the development of hypertension in the adult offspring of diabetic mothers, reinforcing the concept of fetal programming of chronic diseases.

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Author contributions

D. B. Q., F. E. X., J. B.-R. and G. B. conceived and designed the experiments. D. B. Q., E. S., L. C. and M. C. performed the experiments. D. B. Q., E. S., L. C., M. C. and J. B.-R. analysed the data. F. E. X., J. B.-R. and G. B. contributed reagents/materials/analysis tools. D. B. Q., E. S., F. E. X., G. B. and J. B.-R. wrote the manuscript. D. B. Q. and E. S. contributed equally to the study and should be considered joint first authors.

Conflict of interest

None.

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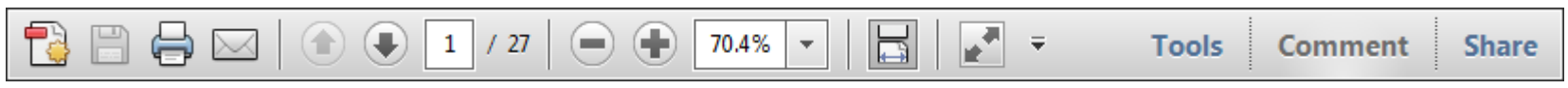

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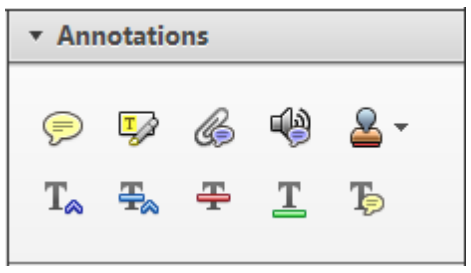

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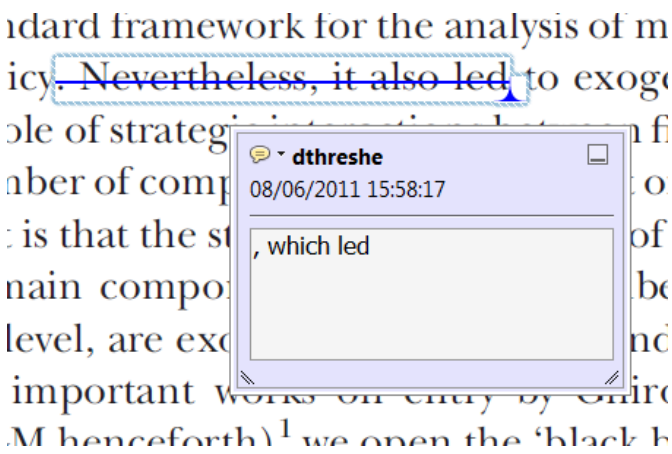

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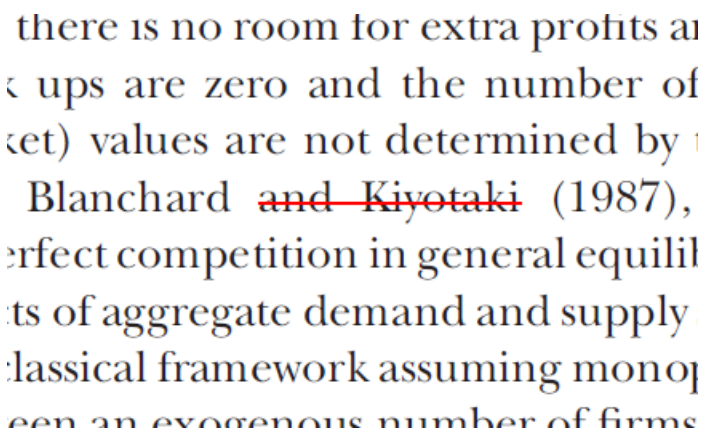

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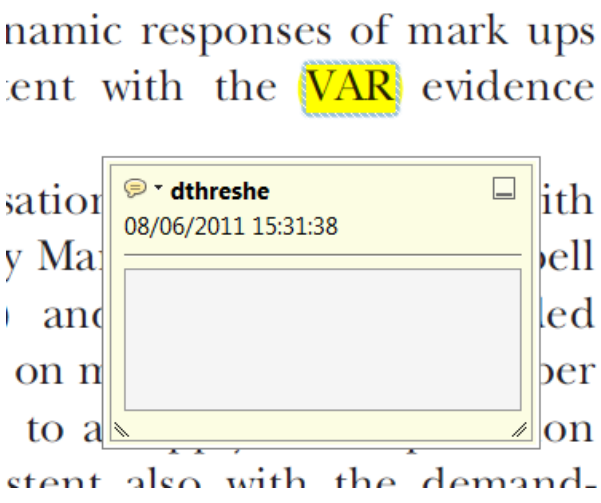

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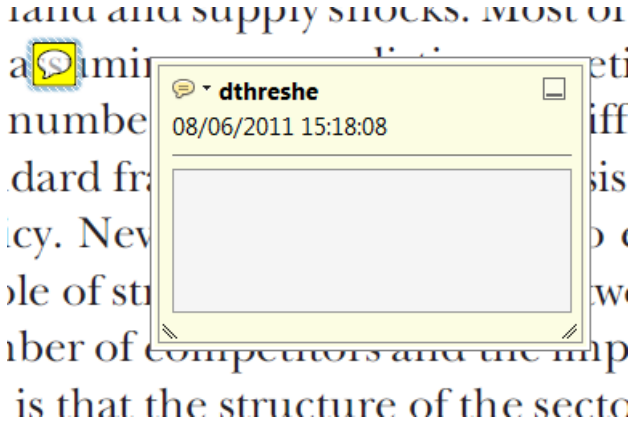

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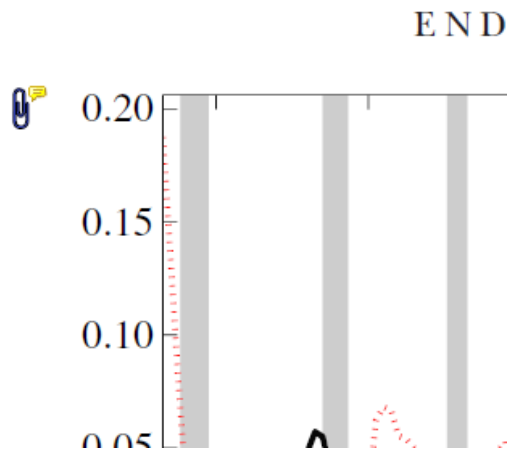

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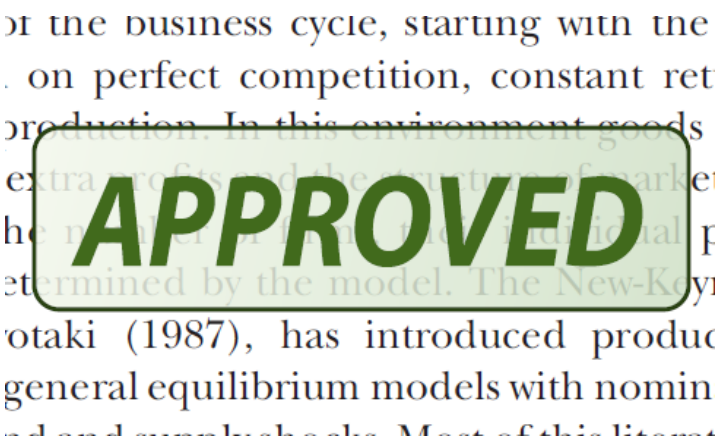

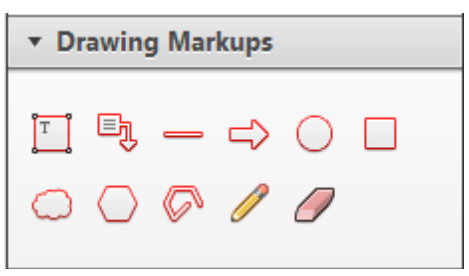

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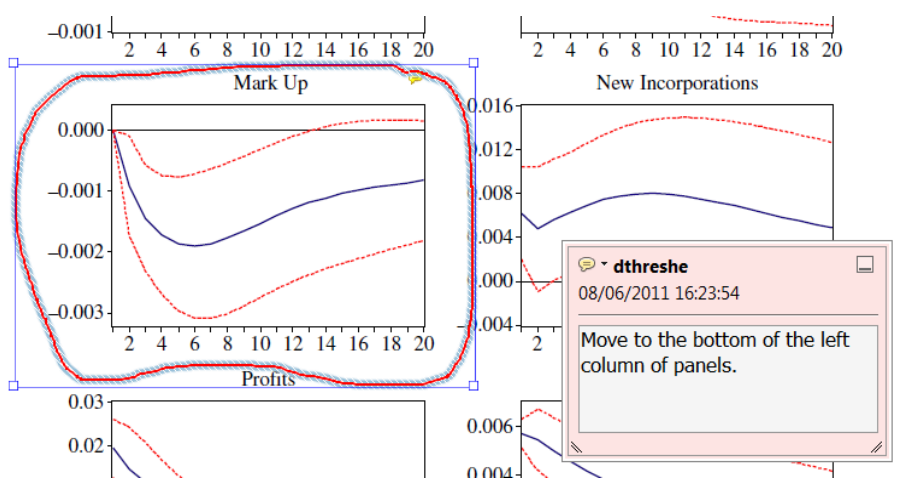

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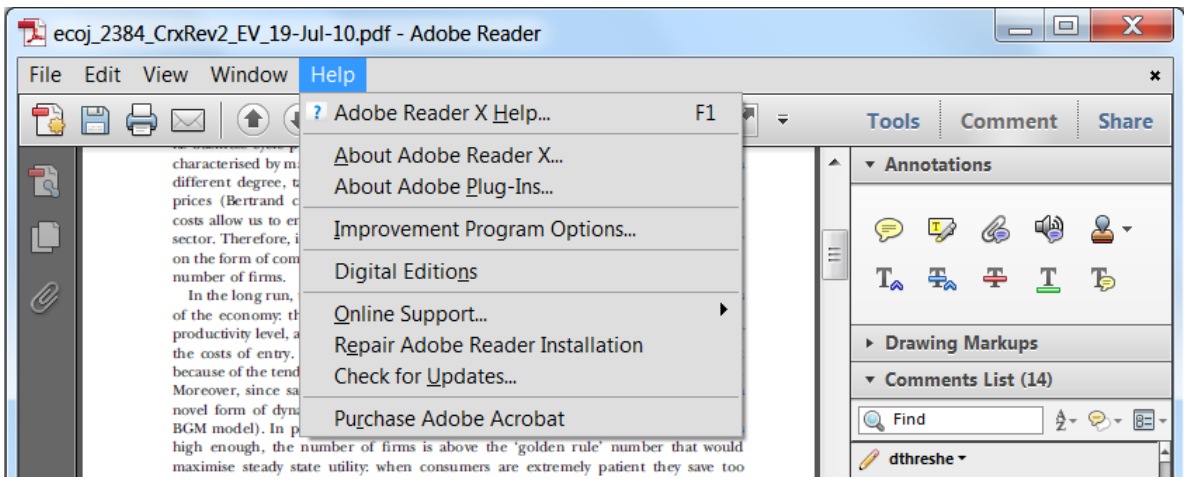

Artículo 5: Aerobic exercise training increases neuronal nitric oxide release and bioavailability and decreases noradrenaline release in mesenteric artery from spontaneously hypertensive rats.

Blanco-Rivero J, Roque FR, **Sastre E**, Caracuel L, Couto GK, Avendaño MS, Paula SM, Rossoni LV, Salaices M, Balfagón G.

J Hypertens. 2013 May;31(5):916-26.

Original Article

Aerobic exercise training increases neuronal nitric oxide release and bioavailability and decreases noradrenaline release in mesenteric artery from spontaneously hypertensive rats

Javier Blanco-Rivero[a,b], Fernanda R. Roque[b,c], Esther Sastre[a,b], Laura Caracuel[a,b], Gisele K. Couto[d], María Soledad Avendaño[b,c], Suliana M. Paula[d], Luciana V. Rossoni[d], Mercedes Salaices[b,c], and Gloria Balfagón[a,b]

Objective: To study the effect of aerobic exercise training on sympathetic, nitrergic and sensory innervation function in superior mesenteric artery from spontaneously hypertensive rats (SHRs).

Methods: De-endothelized vascular rings from sedentary and trained SHRs (treadmill 12 weeks) were used. Vasomotor responses to electrical field stimulation (EFS), noradrenaline, nitric oxide donor DEA-NO and calcitonin gene-related peptide (CGRP) were studied. Neuronal nitric oxide synthase (nNOS) expression and nitric oxide, superoxide anions ($O_2^{\cdot-}$), noradrenaline and CGRP levels were also determined.

Results: Aerobic exercise training decreased vasoconstrictor response to EFS but increased noradrenaline response. Phentolamine decreased while N^{ω}-nitro-L-arginine methyl ester (L-NAME) increased the response to EFS; the effect of both drugs was greater in trained animals. Training also decreased noradrenaline release and $O_2^{\cdot-}$ production and increased nNOS expression, nitric oxide release and the vasodilator response to DEA-NO. The $O_2^{\cdot-}$ scavenger tempol increased DEA-NO-induced vasodilation only in sedentary rats. The EFS-induced contraction was increased to a similar extent in both experimental groups by preincubation with CGRP (8–37). CGRP release and vasodilator response were not modified by training.

Conclusion: Aerobic exercise training decreases contractile response to EFS in mesenteric artery from SHRs. This effect is the net result of decreased noradrenaline release, increased sensitivity to the vasoconstrictive effects of noradrenaline and increased neuronal nitric oxide release and bioavailability. These modifications might contribute to the beneficial effects of aerobic exercise training on blood pressure.

Keywords: aerobic exercise, hypertension, neuronal nitric oxide, nitrergic innervation, noradrenaline, rat mesenteric artery, sympathetic innervation

Abbreviations: 6-OHDA, 6-hydroxydopamine; CGRP, calcitonin gene-related peptide; DEA-NO, diethylamine NONOate; EFS, electric field stimulation; KHS, Krebs–Henseleit solution; L-NAME, N nitro-L-arginine methyl ester; nNOS, neuronal nitric oxide synthase; $O_2^{\cdot-}$, superoxide anions; TTX, tetrodotoxin

INTRODUCTION

Evidence from epidemiological prospective follow-up studies indicates that physical activity and physiological fitness are inversely related to the incidence of cardiovascular disease and mortality. Modifications in lifestyle, including regular exercise, are effective in the management of hypertension [1] with differences in the efficiency of the effect depending on levels and kinds of physical activity. However, the mechanisms underlying the beneficial cardiovascular effects produced by regular exercise are little known.

Vascular tone is determined by an equilibrium among several mechanisms in which perivascular innervation plays an important role. This regulation involves sympathetic, cholinergic, nitrergic, peptidergic and/or sensory innervations that are specific to the vascular bed considered [2,3]. Superior mesenteric artery regulates around 20% of total blood flow. Therefore, changes in mesenteric vascular tone participate in total peripheral resistance. The application of electrical field stimulation (EFS) produces a vasoconstrictor response which is the integrated result of the release of different neurotransmitters, mainly

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noradrenaline from sympathetic nerve endings, nitric oxide from nitrergic innervation and calcitonin gene-related peptide (CGRP) from sensory nerves [4–7]. Alterations of the functional role of these components in rat mesenteric artery have been associated with several experimental and pathophysiological circumstances including ageing, portal hypertension, liver cirrhosis and diabetes [8–11].

Exercise has been shown to reduce hypertension by decreasing the elevated sympathetic activity in blood vessels by different mechanisms, including pre and post-synaptic mechanisms and control of the central nervous system [12]. Training has been described to improve endothelial nitric oxide release in hypertension through an up-regulation of endothelial nitric oxide synthase expression and an increase in neuronal nitric oxide synthase (nNOS) expression in the central nervous system and rat penile tissues [13,14]. Additionally, growing evidence indicates that exercise also prevents oxidative damage by reducing oxidative stress in hypertension [15–19], thus increasing nitric oxide bioavailability. Regarding the influence of training on sensory innervation, cutaneous sensory function can be preserved by maintaining regular exercise training [20], and changes in the sensory receptors to resistive exercise have been described [21].

Taking into account this information, we consider it important to analyze the effect of aerobic exercise training on the vasomotor response to EFS in mesenteric artery from spontaneously hypertensive rats (SHRs), studying the kinds of innervation, sympathetic, nitrergic or sensory, affected, as well as the mechanisms implicated.

MATERIALS AND METHODS

Animals

Three-month-old male SHRs were obtained from the Animal Quarters of the Universidad Autónoma de Madrid (Registration number EX-021U), in accordance with guidelines 609/86 of the E.E.C., R.D. 233/88 of the Ministerio de Agricultura, Pesca y Alimentación of Spain, and Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health [NIH publication No. 85.23 revised 1985]. All experimental procedures involving animal use were approved by the Ethics Committee of the Universidad Autónoma de Madrid (RD 1201/2005).

Rats were housed at a constant room temperature, humidity and light cycle (12 : 12-h light-dark) and had free access to tap water and standard rat chow *ad libitum*. Body weight was controlled weekly and SBP was measured before the start of the protocol and every 15 days until the end of the protocol using tail-cuff plethysmography.

Exercise training protocol

Animals were divided in two experimental groups: sedentary and trained SHRs. Exercise training was performed on a motor treadmill (Motor-driven Treadmill LI8706; Letica Scientific Instruments, Barcelona, Spain) for 12 weeks, 5 times per week for 60 min, gradually progressing toward 55–65% of maximal running speed (15–20 m/min), as described elsewhere [22]. To determine the maximal exercise capacity rats were subjected to a progressive exercise test on a treadmill using an incremental speed protocol of 5 m/min every 3 min and no grade until exhaustion. The treadmill exercise test was repeated after 6 weeks of exercise training in order to adjust training intensity. Rats were considered to be exhausted when they could no longer run at the treadmill speed. The sedentary rats were handled at least twice a week for habituation to the experimental protocols.

Tissue samples

At the end of the experimental protocol and 24 h after the last session of exercise training rats were euthanized by decapitation. The muscle soleus was removed and immediately stored at −80°C until citrate synthase analysis was performed. The first branch of the mesenteric artery was carefully dissected, cleaned out of connective tissue, divided into 2-mm long segments and maintained in cold (4°C) Krebs–Henseleit solution (KHS) (in mmol/l: 115 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4.7H_2O$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 11.1 glucose, and 0.01 Na_2EDTA) bubbled with a 95% O_2–5% CO_2 mixture. For protein expression analysis, some segments were quickly frozen in liquid nitrogen and maintained at −80°C until the day the experiments were performed.

Citrate synthase activity

Citrate synthase activity was determined in mixed right soleus according to the method of Alp *et al.* [23] and used as a marker of muscle oxidative activity. The enzyme activity was measured in whole muscle homogenates, and the complex resulting from acetyl-CoA and oxaloacetate was determined at 412 nm and 25°C, at an interval of 10 min. Citrate synthase activity was expressed as nmol/min per mg of protein.

Vascular reactivity

The method used for isometric tension recording has been described in full elsewhere [7]. Two parallel stainless steel pins were introduced through the lumen of the vascular segment: one was fixed to the bath wall, and the other connected to a force transducer (Grass FTO3C; Quincy, Massachusetts, USA); this, in turn, was connected to a model 7D Grass polygraph. For EFS experiments, segments were mounted between two platinum electrodes 0.5 cm apart and connected to a stimulator (Grass, model S44) modified to supply adequate current strength. Segments were suspended in an organ bath containing 5 ml of KHS at 37°C and continuously bubbled with a 95% O_2 to 5% CO_2 mixture (pH of 7.4). Experiments were performed in endothelium-denuded segments to eliminate the main source of vasoactive substances, including endothelial nitric oxide. This avoided possible actions by different drugs on endothelial cells that could lead to misinterpretation of results. Endothelium was removed by gently rubbing the luminal surface of the segments with a thin wooden stick. The segments were subjected to a tension of 0.5*g*, which was readjusted every 15 min during a 90-min equilibration period before drug administration. After this, the vessels were exposed to 75 mmol/l KCl, to check their functional integrity. Endothelium removal did not alter the contractions elicited by KCl. After a washout period, the absence of

vascular endothelium was tested by the inability of 10 μmol/l acetylcholine (ACh) to relax segments precontracted with noradrenaline.

Frequency–response curves to EFS (1, 2, 4, 8 and 16 Hz) were performed. The parameters used for EFS were 200 mA, 0.3 ms, 1–16 Hz, for 30 s with an interval of 1 min between each stimulus, the time required to recover basal tone. A washout period of at least 1 h was necessary to avoid desensitization between consecutive curves. Two successive frequency-response curves separated by 1-h intervals produced similar contractile responses. To evaluate the neural origin of the EFS-induced contractile response, the nerve impulse propagation blocker, tetrodotoxin (TTX, 0.1 μmol/l) was added to the bath 30 min in advance.

To determine the participation of the adrenergic component of sympathetic innervation in the EFS-induced response in segments from sedentary and trained rats, 1 μmol/l phentolamine, an α-adrenoceptor antagonist, was added to the bath 30 min before performing the frequency–response curve. Additionally, the vasoconstrictor response of exogenous noradrenaline (1 nmol/l–10 μmol/l) was tested in segments from both experimental groups.

The method used to deplete sympathetic innervation has been used previously by our group [24]. Briefly, endothelium-denuded mesenteric segments from both sedentary and trained SHRs were incubated at room temperature for 10 min in KHS ($NaHCO_3$ and NaH_2PO_4 were omitted, unbuffered solution) containing 0.02 mmol/l glutathione and 1.46 mmol/l of the neurotoxin 6-hydroxydopamine (6-OHDA). The pH of this solution was adjusted to 4.9 with 0.05 mmol/l NaOH and then the solution was covered with paraffin oil. Subsequently, the arteries were immersed in normal KHS and EFS-induced contraction experiments were performed.

To analyze the participation of nitric oxide in the EFS-induced response in segments from both groups of rats, 0.1 mmol/l N^{ω}-nitro-L-arginine methyl ester (L-NAME), a nonspecific inhibitor of NOS, was added to the bath 30 min before performing the second frequency–response curve. The vasodilator response to the nitric oxide donor, diethylamine NONOate (DEA-NO; 0.1 nmol/l–0.1 mmol/l) was determined in noradrenaline-precontracted arteries from both experimental groups. Some experiments were performed in the presence of superoxide anion scavenger tempol (0.1 mmol/l).

To study the possible participation of sensory innervation in the EFS-induced response, 0.5 μmol/l CGRP (8–37), a CGRP receptor antagonist, was added to the bath 30 min before performing the second frequency–response curve. Additionally, the vasodilator response to exogenous CGRP (0.1 nmol/l–0.1 μmol/l) was determined in noradrenaline-precontracted arteries from both experimental groups.

Noradrenaline and calcitonin gene-related peptide levels

Noradrenaline and CGRP releases were measured using commercial kits (Noradrenaline research EIA Kit; Labor Diagnostica Nord, Gmbh and Co., KG, Nordhon, Germany, and Rat CGRP Enzyme Immunoassay Kit; Spibio, Bertin Group) following the manufacturer's instructions. Endothelium-denuded segments of rat mesenteric arteries from both experimental groups were preincubated for 30 min in 5 ml of KHS at 37°C and continuously gassed with a 95% O_2–5% CO_2 mixture (stabilization period). This was followed by two washout periods of 10 min in a bath of 0.4 ml of KHS. Then, the medium was collected to measure basal release. Afterwards, the organ bath was refilled, and cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz were applied at 1-min intervals. Afterwards, the medium was collected to measure EFS-induced neurotransmitter release. Samples were immediately frozen in liquid nitrogen and conserved at −70°C until experiments were performed. To prevent catecholamine degradation, 1 mmol/l EDTA and 4 mmol/l sodium metabisulfite were added to the samples. Additionally, endothelium-denuded mesenteric segments from sedentary and trained SHRs were collected in order to measure noradrenaline content in the vessel segments. Samples were homogenized in 0.01 N HCl containing 1 mmol/l EDTA and 4 mmol/l sodium metabisulfite, as the manufacturer suggests. Results were expressed as ng noradrenaline/ml mg tissue (medium measurements), ng noradrenaline/mg protein (tissue measurements) and pg CGRP/ml mg tissue.

Nitric oxide measurements

Nitric oxide release was measured using fluorescence emitted by the fluorescent probe 4,5-diaminofluorescein (DAF-2), as previously described [25,26]. Endothelium-denuded mesenteric arteries from sedentary and trained SHRs were subjected to a 60-min equilibration period in HEPES buffer (in mmol/l: NaCl 119; HEPES 20; $CaCl_2$ 1.2; KCl 4.6; $MgSO_4$ 1; KH_2PO_4 0.4; $NaHCO_3$ 5; glucose 5.5; Na_2HPO_4 0.15; pH 7.4) at 37°C. Arteries were incubated with 2 μmol/l DAF-2 for 30 min. The medium was then collected to measure basal nitric oxide release. Once the organ bath was refilled, cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz at 1-min intervals were applied. Afterwards, the medium was collected to measure EFS-induced nitric oxide release. The fluorescence of the medium was measured at room temperature using a spectrofluorometer (LS50 Perkin Elmer Instruments; FL WINLAB Software, Waltham, Massachusetts, USA) with excitation wavelength set at 492 nm and emission wavelength at 515 nm.

The EFS-induced nitric oxide release was calculated by subtracting basal nitric oxide release from that evoked by EFS. Also, blank sample measures were collected in the same way from segment-free medium in order to subtract background emission. Some assays were performed in the presence of either TTX (0.1 μmol/l), the specific nNOS inhibitor 7-NI (0.1 mmol/l) or the specific iNOS inhibitor 1400 W (1 μmol/l) [9,27,28]. The amount of nitric oxide released was expressed as arbitrary units/mg tissue.

Superoxide anions measurements

Superoxide anion levels were measured using lucigenin chemiluminescence, as previously described [26]. Endothelium-denuded mesenteric segments from both groups

of rats were rinsed in KHS for 30 min, equilibrated for 30 min in HEPES buffer at 37°C, transferred to test tubes that contained 1 ml HEPES buffer (pH 7.4) containing lucigenin (5 μmol/l) and then kept at 37° C. The luminometer was set to report arbitrary units of emitted light; repeated measurements were collected for 5 min at 10-s intervals and averaged. 4,5-Dihydroxy-1,3-benzenedisulphonic acid 'Tiron' (10 mmol/l), a cell-permeant, non-enzymatic superoxide anion scavenger, was added to quench the superoxide anion-dependent chemiluminescence. Calculations were performed subtracting the lucigenin chemiluminescence obtained in the presence of Tiron from that obtained in its absence. Blank measures were collected in the same way without mesenteric segments to subtract background emission.

Neuronal nitric oxide synthase expression

Western blot analysis of nNOS expression was performed as previously described [25]. For these experiments, we used mouse monoclonal nNOS antibody (1 : 1000; Transduction Laboratories) and monoclonal antiβ-actin-peroxidase antibody (1 : 50000; Sigma–Aldrich, Spain). Rat brain homogenates were used as positive control.

Drugs used

L-NA hydrochloride, ACh chloride, diethylamine NONOate diethilammonium salt, CGRP, CGRP (8–37), TTX, L-NAME hydrochloride, 7NI, 1400 W, phentolamine, lucigenin, Tiron, 6-OHDA and DAF-2 (Sigma-Aldrich, Madrid, Spain) were used. Stock solutions (10 mmol/l) of drugs were made in distilled water, except for noradrenaline, which was dissolved in a NaCl (0.9%)-ascorbic acid (0.01% w/v) solution. These solutions were kept at −20°C and appropriate dilutions were made in KHS on the day of the experiment.

Data analysis

The responses elicited by EFS and noradrenaline were expressed as a percentage of the initial contraction elicited by 75 mmol/l KCl for comparison between sedentary and trained rats. The relaxation induced by DEA-NO or CGRP was expressed as a percentage of the initial contraction elicited by noradrenaline (sedentary: 1124.2 ± 6.7 mg; trained: 1115.9 ± 7.4 mg; $P > 0.05$). Results are given as mean ± SEM. Statistical analysis was done by comparing the curve obtained in the presence of the different substances with the control curve by means of ANOVA for repeated measures, followed by the Bonferroni post-hoc test, using the GraphPad Prism 5.0 software (San Diego, California, USA). For nitric oxide, superoxide anion and noradrenaline release data, the statistical analysis was done using one-way ANOVA followed by the Newman–Keuls post-hoc test. P less than 0.05 was considered significant.

RESULTS

Animal evolution

No differences in body weight gain were observed between sedentary and trained SHRs either before or after training [sedentary: 74.9 ± 13.6 g ($n = 7$); trained: 74.6 ± 9.5 g ($n = 15$)]. Blood pressure was significantly decreased by training (sedentary: 219.4 ± 5.0 mmHg; trained: 193.8 ± 1.8 mmHg; $P < 0.05$; $n = 6$ animals each group). Citrate synthase activity in mixed soleus was significantly higher in trained SHRs compared to sedentary animals (sedentary: $91.8.4 \pm 3.7$ nmol/min mg protein; trained: 127.2 ± 3.6 nmol/min mg protein; $P < 0.05$; $n = 5$ animals per group).

Vascular responses to electrical field stimulation

In endothelium-denuded mesenteric segments, the vasoconstrictor response to 75 mmol/l KCl was similar in both experimental groups (sedentary: 722.6 ± 45.64 mg; trained: 723.3 ± 28.23 mg; $P > 0.05$). The application of EFS induced a frequency-dependent contractile response in segments from both experimental groups. This vasoconstriction was lower in segments from trained rats (Fig. 1a). EFS-induced contractions were practically abolished in segments from both experimental groups by the nerve impulse blocker TTX (0.1 μmol/l), indicating the neuronal origin of the factors that induce this response (Fig. 1 b and 1c)

Effect of training on sympathetic component of vascular responses to electrical field stimulation

Preincubation with the α-adrenergic antagonist phentolamine (1 μmol/l) decreased the vasoconstrictor response in segments from both experimental groups, but to a greater extent in segments from sedentary rats (Fig. 2a and 2b, Table 1). The remnant vasoconstriction induced by EFS after preincubation with phentolamine was similar in both experimental groups (Fig. 2a and 2b). Preincubation with 6-OHDA abolished EFS-induced contraction in segments from both experimental groups (Table 1). The contraction elicited by exogenous noradrenaline (1 nmol/l–10 μmol/l) was increased in segments from trained rats (Fig. 2c). Both basal and EFS-induced noradrenaline releases were decreased in segments from trained rats (Fig. 2d). Training did not modify noradrenaline content in mesenteric segments (sedentary: 187.4 ± 11.7 ng noradrenaline/mg protein; trained: 200.3 ± 25.5 ng noradrenaline/mg protein; $P > 0.05$; $n = 9$ animals each group).

Effect of training on nitrergic component of vascular responses to electrical field stimulation

Preincubation with unspecific NOS inhibitor L-NAME (0.1 mmol/l) significantly increased the EFS-contractile response in segments from both experimental groups. This increase was greater in segments from trained animals (Fig. 3a and 3b, Table 1).

Neuronal NOS expression was increased in segments from trained rats compared to sedentary rats (Fig. 4a). Training did not modify basal nitric oxide release. However, EFS-induced nitric oxide release was significantly greater in segments from trained rats than those from sedentary animals (Fig. 4b). Preincubation with 0.1 μmol/l TTX or 0.1 mmol/l of the specific nNOS inhibitor 7-NI abolished EFS-induced nitric oxide release, whereas preincubation

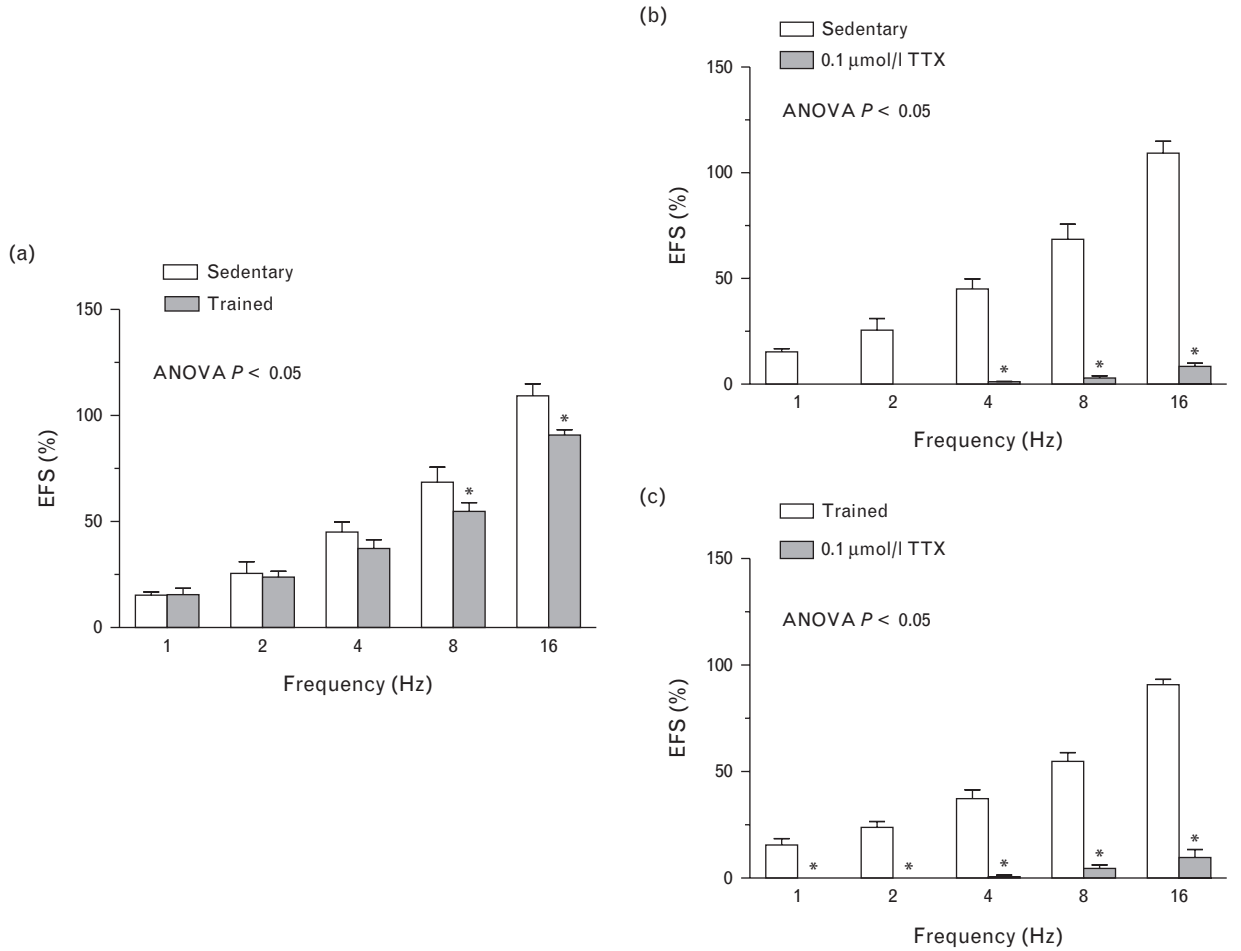

FIGURE 1 Vasoconstrictor response to EFS in mesenteric segments from sedentary and trained SHRs. Results (mean ± SEM) were expressed as a percentage of the initial contraction elicited by KCl. $^{*}P < 0.05$ vs. sedentary animals for each frequency (Bonferroni test). $n = 10$ animals each group. Effect of preincubation with 0.1 μmol/l TTX on the vasoconstrictor response induced by EFS in mesenteric segments from sedentary (b) and trained SHRs (c). Results (mean ± SEM) are expressed as a percentage of the previous contraction elicited by KCl. $n = 6$–8 animals each group. $^{*}P < 0.05$ vs. conditions without specific inhibitor for each frequency (Bonferroni test). EFS, electrical field stimulation; SHR, spontaneously hypertensive rat; TTX, tetrodotoxin.

with 1 μmol/l of the specific iNOS inhibitor 1400 W did not modify it (Fig. 4c and 4d).

In segments precontracted with noradrenaline (sedentary: 1124.2 ± 6.7 mg; trained: 1105.9 ± 7.4 mg; $P > 0.05$), vasodilator response elicited by exogenous nitric oxide donor DEA-NO (0.1 nmol/l–10 μmol/l) was greater in mesenteric segments from trained rats than sedentary animals (Fig. 5a). Preincubation with the superoxide anion scavenger Tempol (0.1 mmol/l) increased vasodilator response to DEA-NO only in segments from sedentary rats (Fig. 5b and 5c). Superoxide anion formation was lower in segments from trained rats (Fig. 5d).

Effect of training on sensory component of vascular responses to electrical field stimulation

Preincubation with the CGRP receptor antagonist CGRP (8–37) (0.5 μmol/l) increased the EFS-induced contraction to a similar extent in segments from both experimental groups (Fig. 6a and 6b, Table 1). Additionally, in noradrenaline-precontracted mesenteric segments (sedentary: 1124.2 ± 6.7 mg; trained: 1105.9 ± 7.4 mg; $P > 0.05$), the vasodilator response to exogenous CGRP was similar in segments from both sedentary and trained SHRs (Fig. 6c), as were both basal and EFS-induced CGRP release (Fig. 6d)

DISCUSSION

The present study shows that aerobic exercise training diminished the vasoconstrictor response due to stimulation of perivascular innervation in mesenteric artery from SHRs, possibly contributing to the decreased blood pressure observed after training. The decreased vasoconstrictor response is due to a reduction in sympathetic innervation function mediated by decreased noradrenaline release, combined with the increased nitrergic function produced by increased nitric oxide release, as well as a possible decrease in nitric oxide scavenging by reactive oxygen species.

In mesenteric rings from both sedentary and trained SHRs, EFS produced a frequency-dependent contraction that was abolished in the presence of TTX, indicating that this vasoconstriction is the result of neurotransmitter release

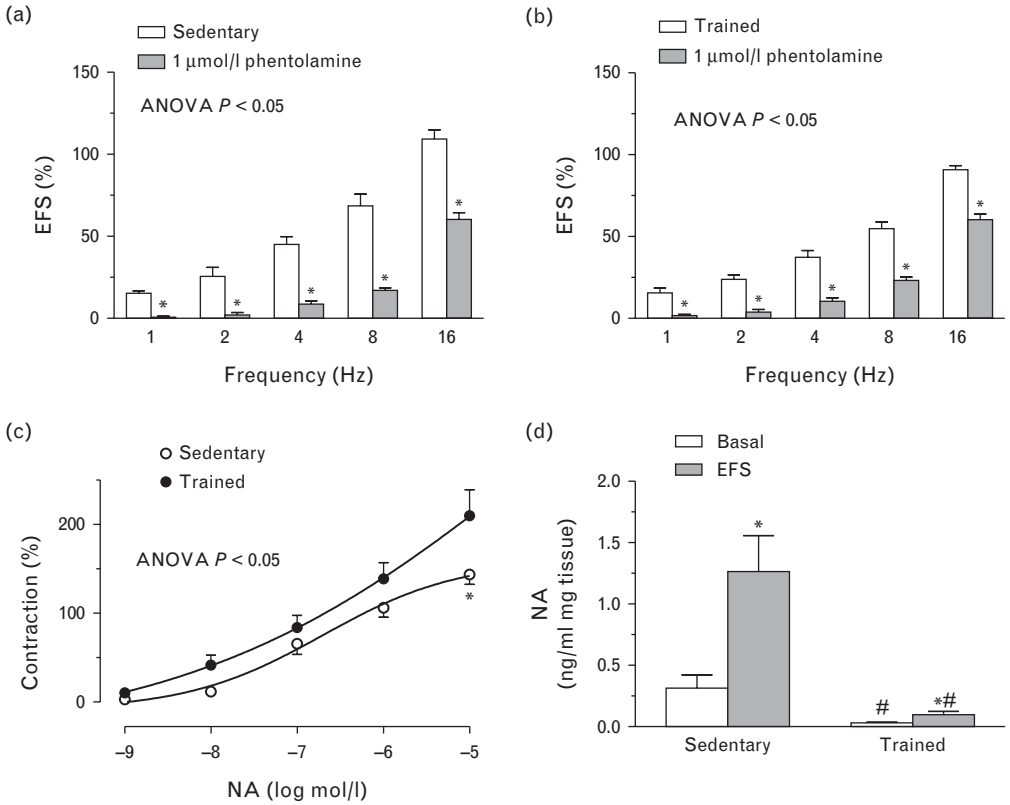

FIGURE 2 Effect of preincubation with 1 μmol/l phentolamine on the vasoconstrictor response induced by EFS in mesenteric segments from sedentary (a) and trained SHRs (b). Results (mean ± SEM) are expressed as a percentage of the previous contraction elicited by KCl. $n = 6–10$ animals each group. $^*P < 0.05$ vs. conditions without specific inhibitor for each frequency (Bonferroni test). (c) Vasoconstrictor response to exogenous NA in segments from sedentary and trained SHRs. Results (mean ± SEM) are expressed as a percentage of the previous contraction elicited by KCl. $^*P < 0.05$ vs. control conditions for each dose (Bonferroni test). $n = 6–10$ animals each group. (d) Basal and EFS-induced NA release in mesenteric segments from sedentary and trained SHR. Results (mean ± SEM) are expressed as ng NA/ml mg tissue. $^*P < 0.05$ vs. basal; # $P < 0.05$ vs. sedentary. $n = 6–10$ animals each group. EFS, electrical field stimulation; NA, noradrenaline; SHR, spontaneously hypertensive rat; TTX, tetrodotoxin.

from perivascular nerves. KCl vasoconstriction is an indicator of the integrity of intrinsic contractile machinery. Previous reports have shown a lower KCl vasoconstriction in resistance arteries isolated from exercise trained rats [29,30], whereas others have found no differences [31]. This discrepancy could be attributed to differences in the vascular bed analyzed and/or kind of training. The fact that in our experimental conditions the vasoconstrictor response to KCl remained unmodified by training indicates the maintenance of the capacity for vasoconstriction, and suggests that the decrease observed in the EFS-induced contraction in trained rats could be associated with changes in neurotransmitter/s release and/or vasomotor response.

Sympathetic, sensory and nitrergic innervation are involved in the vasomotor response to EFS in rat mesenteric artery [4–7]. Hypertension is known to be particularly dependent on the activity of the sympathetic system [2,32] and training can either increase or decrease

TABLE 1. EFS inhibition after preincubation with 1 μmol/l phentolamine or 1.46 mmol/l 6-OHDA, and EFS potentiation after preincubation with 0.1 mmol/l L-NAME or 0.5 μmol/l CGRP (8–37) in mesenteric segments from sedentary and trained SHRs

	1 Hz	2 Hz	4 Hz	8 Hz	16 Hz
Sedentary + phentolamine	98.6 ± 4.4	98.3 ± 1.7	86.6 ± 5.8	77.4 ± 4.5	55.2 ± 3.1
Trained + phentolamine	94.4 ± 4.2	93.2 ± 3.7	73.7 ± 1.1[a]	59.3 ± 2.1[a]	34.1 ± 1.9[a]
Sedentary + 6-OHDA	99.9 ± 0.5	99.8 ± 0.4	99.8 ± 0.3	99.7 ± 0.7	99.5 ± 0.6
Trained + 6-OHDA	99.9 ± 0.1	99.2 ± 0.6	99.9 ± 0.4	99.5 ± 0.8	99.4 ± 0.2
Sedentary + L-NAME	37.2 ± 3.1	33.6 ± 4.5	36.3 ± 214	21.6 ± 3.1	13.5 ± 2.1
Trained + L-NAME	75.7 ± 8.4[a]	81.4 ± 5.4[a]	57.9 ± 5.2[a]	46.3 ± 4.3[a]	24.8 ± 3.2
Sedentary + CGRP (8–37)	44.1 ± 7.2	48.4 ± 9.2	45.7 ± 7.4	31.7 ± 5.3	18.9 ± 3.2
Trained + CGRP(8–37)	38.1 ± 5.5	47.1 ± 7.1	43.5 ± 7.2	26.7 ± 4.6	24.8 ± 5.6

CGRP, calcitonin gene-related peptide; EFS, electrical field stimulation; L-NAME, N^{ω}-nitro-L-arginine methyl ester; SHR, spontaneously hypertensive rat. This table presents the percentage (%) of inhibition/potentiation of EFS-induced contraction after preincubation with 1 μmol/l phentolamine, 1.46 mmol/l 6-OHDA, 0.5 μmol/l CGRP (8–37) or 0.1 mmol/l L-NAME in mesenteric segments from sedentary and trained SHRs. Calculations are performed taking the KCl-induced contraction as 100% of the contractile response. Results are expressed as mean ± SEM.
[a]$P < 0.05$ vs. sedentary. $n = 6–10$ animals each group.

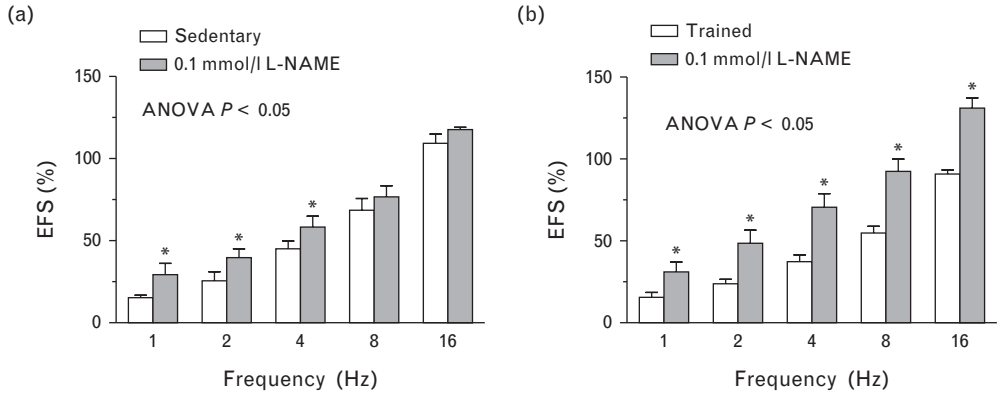

FIGURE 3 Effect of preincubation with 0.1 mmol/l L-NAME on the vasoconstrictor response induced by EFS in mesenteric segments from sedentary (a) and trained SHRs (b). Results (mean ± SEM) are expressed as a percentage of the previous contraction elicited by KCl. $n = 6–10$ animals each group. $^{*}P < 0.05$ vs. conditions without specific inhibitor for each frequency (Bonferroni test). EFS, electrical field stimulation; SHR, spontaneously hypertensive rat.

sympathetic activity, depending on the training program and vascular bed analyzed [33–36]. For this reason, we analyzed the effect of aerobic exercise training on the response to EFS in mesenteric artery preincubated with the nonselective α-adrenergic antagonist, phentolamine. This drug reduced vasoconstrictor response to EFS in segments from both experimental groups, but the effect was less marked in mesenteric segments from trained animals

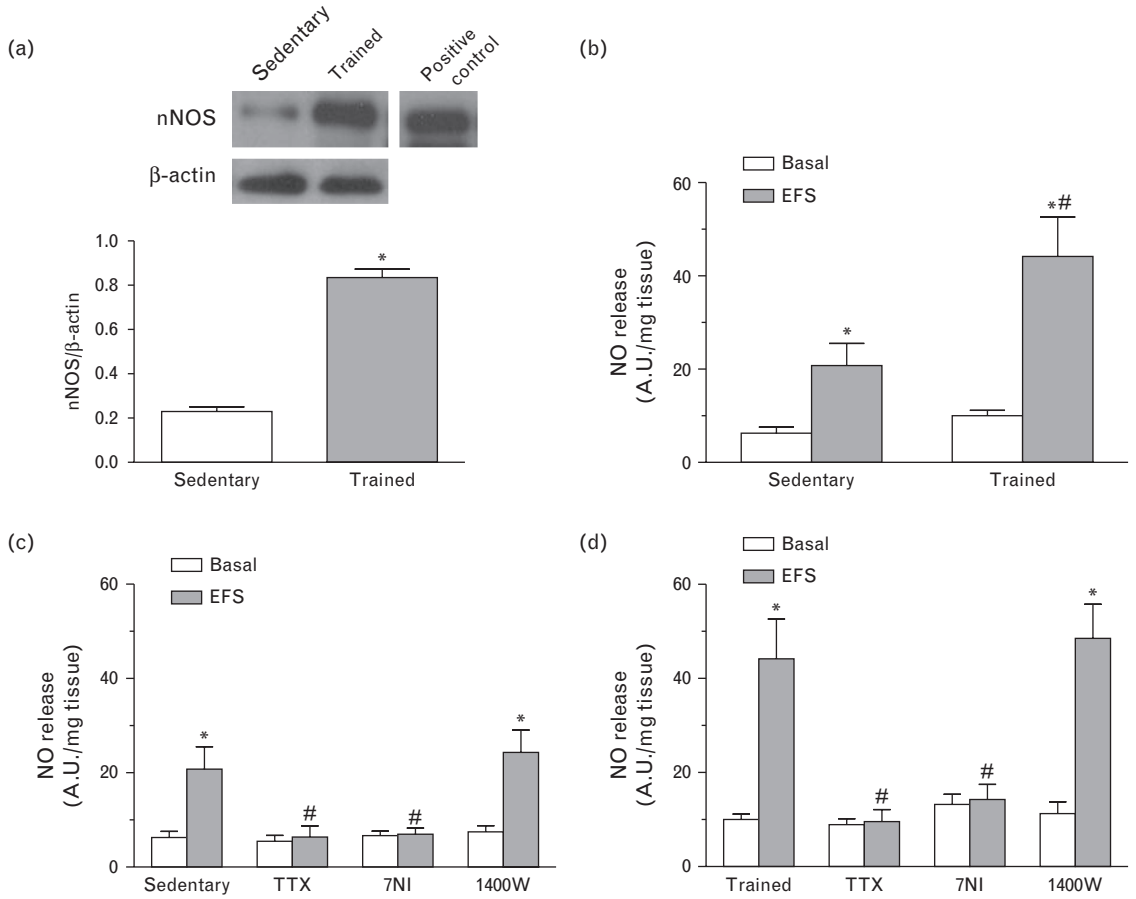

FIGURE 4 (a) Effect of training on nNOS expression. The blot is representative of five separate segments from each group. Lower panel shows densitometric analysis for nNOS expression. Results (mean ± SEM) are expressed as the ratio of the signal obtained for nNOS and that obtained for α-actin. $^{*}P < 0.05$ vs. sedentary. (b) Basal and EFS-induced NO release in mesenteric segments from sedentary and trained SHR. Effect of preincubation with 0.1 μmol/l TTX, 0.1 mmol/l L-NAME or 1 μmol/l 1400 W on basal and EFS-induced NO release in mesenteric segments from sedentary (c) and trained (d) SHR. Results (mean ± SEM) are expressed as arbitrary units (A.U.)/ mg tissue. $^{*}P < 0.05$ vs. basal; # $P < 0.05$ vs. sedentary. $n = 4–7$ animals per group. EFS, electrical field stimulation; L-NAME, N^{ω}-nitro-L-arginine methyl ester; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; SHR, spontaneously hypertensive rat.

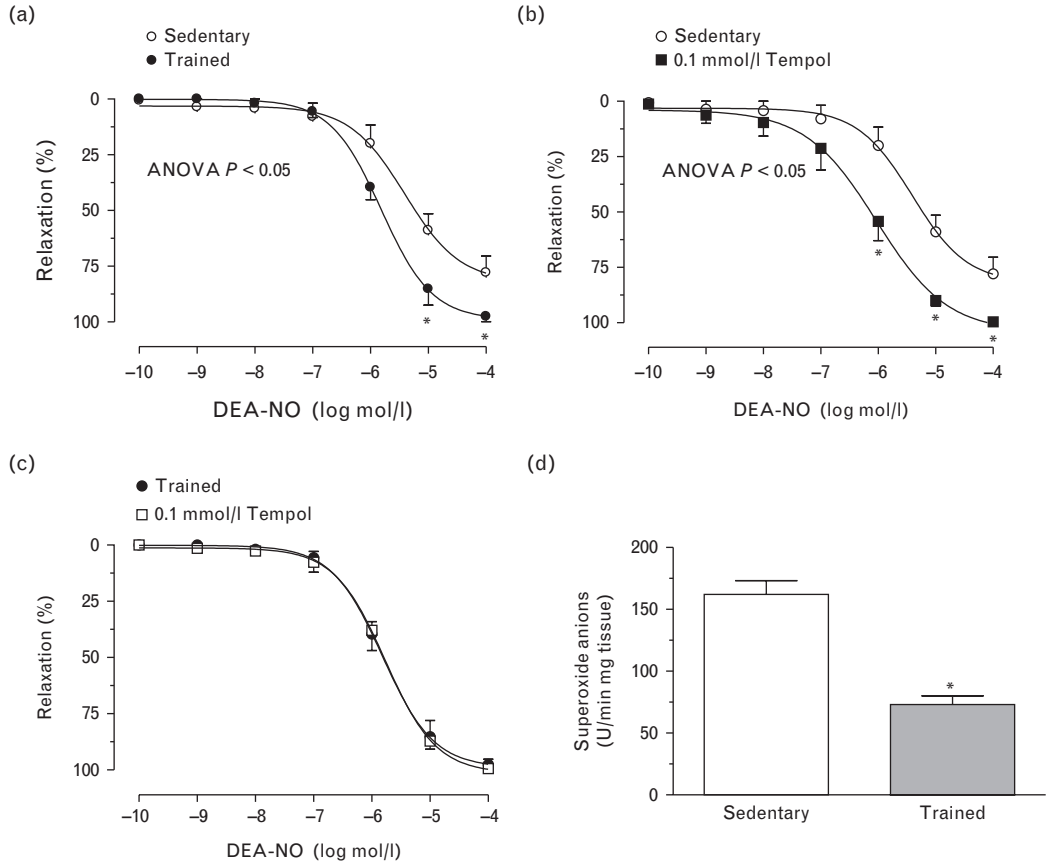

FIGURE 5 (a) Vasodilator response to NO donor DEA-NO in segments from sedentary and trained SHR. Effect of preincubation with 0.1 mmol/l Tempol on the vasodilator response elicited by DEA-NO in segments from sedentary (b) and trained SHRs (c). Results (mean ± SEM) are expressed as a percentage of the initial contraction elicited by exogenous NA. $^*P < 0.05$ vs. control conditions for each dose (Bonferroni test). $n = 7–10$ animals each group. (d) Superoxide anion release in mesenteric segments from sedentary and trained SHRs. Results (mean ± SEM) are expressed as chemiluminiscence units (U)/min mg tissue. $^*P < 0.05$ vs. control. $n = 5$ animals per group. NA, noradrenaline; NO, nitric oxide; SHR, spontaneously hypertensive rat.

than in segments from sedentary SHRs. However, there was a remaining contractile response which was phentolamine-resistant. After preincubation with the neurotoxin 6-OHDA, which depletes sympathetic innervation, EFS-induced contraction was practically abolished, indicating that the EFS-induced contraction is due to neurotransmitters released from sympathetic nerve endings. Additionally, the fact that there were no significant differences between the remnant contractions induced by EFS after pre-incubation with phentolamine in the segments from either group, indicates that training does not modify the vasomotor role of this additional sympathetic component, which could be probably ATP, since we have recently described in this vascular bed that ATP is the sympathetic neurotransmitter responsible of the remnant vasoconstriction obtained after preincubation with phentolamine [25,37].

The different reductions produced by phentolamine in the EFS-induced contractions suggests that a decrease in the role played by the adrenergic component of the sympathetic innervation could be responsible for the decreased vasoconstrictor response to EFS in trained SHRs, as has already been reported in the central nervous system [12]. This effect could be due to a decrease in noradrenaline release and/or the vasoconstrictor response. To determine this we first analyzed noradrenaline release, observing a huge drop in both basal and EFS-induced noradrenaline release in segments from trained SHRs, in agreement with previous reports describing decreases in plasmatic noradrenaline [38]. Regarding the effect of exercise, increase, decrease and no changes in vasoconstrictor response to alpha-adrenergic agonists have been reported [29–31]. In our experimental conditions, we observed that aerobic exercise training increased the vasoconstrictor response to noradrenaline, possibly due to an up-regulation process consequent to the decrease in noradrenaline release. Although both results indicate opposite effects of training on sympathetic function, the net effect is a decreased role for sympathetic innervation in mesenteric segments from trained SHRs. Additionally, the fact that noradrenaline levels were similar in arteries from sedentary and trained SHRs confirms that the reduction in sympathetic function is due to the decreased noradrenaline release from adrenergic innervation, and not to modifications in vessel noradrenaline content. The diminished sympathetic innervation function could, by itself, explain the decreased EFS vasoconstriction observed in mesenteric segments from trained SHRs. However, the participation of other neural components cannot be ruled out.

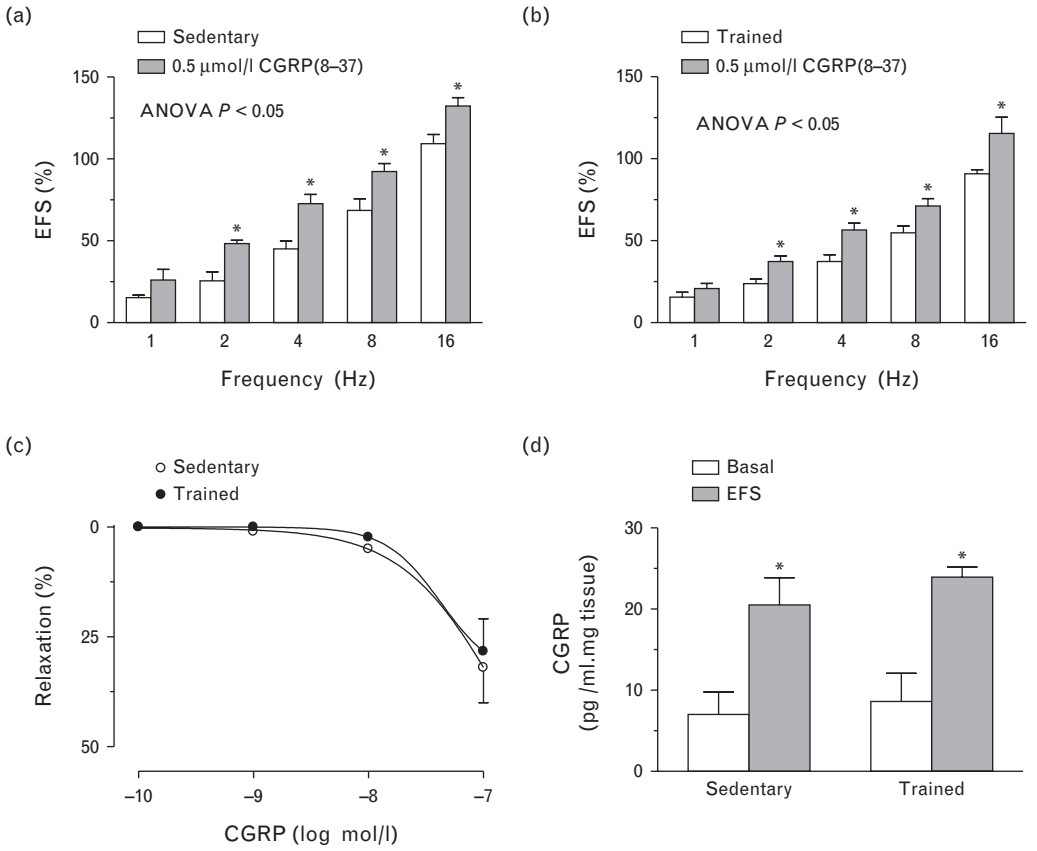

FIGURE 6 (a) Effect of preincubation with 0.5 μmol/l CGRP (8–37) on the vasoconstrictor response induced by EFS in mesenteric segments from sedentary (a) and trained (b) SHRs. Results (mean + SEM) are expressed as a percentage of previous contraction elicited by KCl. $^*P < 0.05$ vs. conditions without specific inhibitor for each frequency (Bonferroni test). $n = 6–10$ animals each group. (c) Vasodilator response to exogenous CGRP in segments from sedentary and trained SHR. Results (mean ± SEM) are expressed as a percentage of a previous contraction elicited by NA. $n = 6–10$ animals each group. (d) Basal and EFS-induced CGRP release in mesenteric segments from sedentary and trained SHRs. Results (mean ± SEM) are expressed as pg CGRP/ml mg tissue. $^*P < 0.05$ vs. basal. $n = 6–10$ animals per group. CGRP, calcitonin gene-related peptide; SHR, spontaneously hypertensive rat, NA, noradrenaline.

The nitric oxide released from nitrergic innervation by EFS has been demonstrated to have a vasodilator role contributing to the vasomotor response to EFS [7,11,39–41]. In vascular reactivity experiments, preincubation with 7-NI decreases vasoconstrictor response to noradrenaline [25,42], which could lead to misinterpretation of the results. That is why we used the nonselective NOS inhibitor L-NAME in the analysis of vasomotor response to EFS. Vasoconstriction induced by EFS was increased in segments from both groups of rats incubated with L-NAME, confirming an involvement by nitric oxide in the EFS-induced response. The greater effect of L-NAME in segments from trained rats suggests an increased role of neuronal nitric oxide in EFS-induced vasoconstriction, possibly related to increases in nitric oxide release and/or increases in the vasodilator response. We observed that EFS increased nitric oxide release, in agreement with previous reports [43,44], and that exercise training increased this release. The EFS-induced nitric oxide release was abolished by TTX in both groups, thus confirming the neural origin of the nitric oxide, and neuronal nitric oxide could participate in the effect on the EFS-contraction produced by training. nitric oxide production in neural tissue can have two sources: nNOS and iNOS [45,46]. In the present study, the specific nNOS inhibitor 7-NI, but not the iNOS inhibitor 1400 W, decreased EFS-induced nitric oxide release, indicating that nitric oxide release is dependent on nNOS. The increased nitric oxide release from nNOS correlates with the increased nNOS expression observed in mesenteric segments from trained SHRs. In agreement with this possibility, an increase in nNOS through training has been reported in the central nervous system and skeletal muscle [13,47,48].

Moderate exercise training improves the vascular antioxidant defense systems, increasing nitric oxide bioavailability [15,19,49]. We observed that superoxide anion production is decreased in mesenteric segments from trained SHR and that the superoxide anion scavenger tempol increased the vasodilator response to DEA-NO only in segments from sedentary rats. In addition, the vasodilator response to DEA-NO was greater in segments from trained than from sedentary rats. Taken together these results suggest that increased neuronal nitric oxide release and bioavailabiity through diminished superoxide anion generation could be an important mechanism by which aerobic exercise mediates the beneficial vascular effects in hypertension.

Once we had analyzed the participation of sympathetic and nitrergic innervation in the diminished response to EFS in mesenteric segments from trained SHRs, we studied the possible role of sensory innervation in this vasomotor

response, since exercise has been shown to modulate the role of sensory innervation in several tissues [20]. Preincubation with the CGRP receptor antagonist CGRP (8–37) increased EFS-induced vasoconstriction to a similar extent in both sedentary and trained SHRs. Additionally, CGRP release and vasodilator response to this peptide were also similar in both experimental groups. These observations led us to conclude that training did not alter sensory innervation function.

In conclusion, the present results demonstrate that aerobic exercise training affects the function of vascular innervation in mesenteric artery from SHR, inducing a decreased contractile response to EFS. This effect is the net result of decreased noradrenaline release, increased noradrenaline vasoconstriction and increased neuronal nitric oxide release and bioavailability. Sensory innervation function remains unaltered. These modifications might contribute to the decreased blood pressure in SHRs observed after aerobic exercise training.

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Conflicts of interest

There are no conflicts of interest.

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Reviewer's Summary Evaluations

Referee 1

Exercise training in spontaneously hypertensive rats reduces the reactivity of proximal mesenteric artery segments to perivascular nerve stimulation. It is demonstrated that this is due to NO-dependent reduction in norepinephrine release from sympathetic nerves. These modifications in sympathetic nerve function could contribute to exercise-induced reductions in vascular resistance and arterial pressure. It remains to be clarified to what extent such exercise-induced changes in the neural control of vascular function take place in other vascular beds and in peripheral segments of the arterial tree which are of great importance for regulating organ perfusion.

Artículo 6: Aerobic exercise training increases nitrergic innervation function and decreases sympathetic innervation function in mesenteric artery from rats fed a high-fat diet.

Sastre E, Caracuel L, Balfagón G, Blanco-Rivero J.

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Original Article

Aerobic exercise training increases nitrergic innervation function and decreases sympathetic innervation function in mesenteric artery from rats fed a high-fat diet

Esther Sastre*, Laura Caracuel*, Gloria Balfagón, and Javier Blanco-Rivero

Introduction: We investigated whether high-fat diet (HFD)-induced obesity was associated with modifications in mesenteric innervation function, the mechanisms involved, and the possible effects of aerobic exercise training on these changes.

Materials and methods: :Male Wistar rats were divided into three groups: rats fed a standard diet (control group); rats fed a HFD (35% fat) for 8 weeks; and HFD rats submitted to aerobic exercise training (8 weeks, 5 times per week for 50 min). Segments of isolated mesenteric arteries were exposed to electric field stimulation (EFS) with or without phentolamine, suramin, or Nω nitro-L-arginine methyl ester. Noradrenaline, ATP, and nitric oxide release, and total and phosphorylated neuronal nitric oxide synthase (nNOS, P-nNOS) expression were also measured.

Results: EFS contraction was greater in sedentary HFD than in control rats. Phentolamine reduced EFS contractions more markedly in HFD rats. Suramin decreased EFS contractions only in control rats. Phentolamine + suramin practically abolished EFS-induced contraction in control rats, whereas it did not modify it in the HFD rats. Noradrenaline release was greater and ATP was lower in HFD rats. Nω nitro-L-arginine methyl ester increased contractions to EFS only in segments from control rats. Nitric oxide release and nNOS and P-nNOS expressions were lower in arterial segments from HFD rats than from control rats. None of these changes in sedentary HFD rats was present in the trained HFD rats.

Conclusions: Enhanced sympathetic and diminished nitrergic components contributed to increased vasoconstrictor responses to EFS in sedentary HFD rats. All these changes were avoided by aerobic exercise training, suggesting that aerobic exercise could reduce peripheral vascular resistance in obesity.

Keywords: aerobic exercise training, nitrergic innervation, obesity, superior mesenteric artery, sympathetic innervation

Abbreviations: CGRP, calcitonin gene-related peptide; DEA-NO, diethylamine NONOate; EFS, electric field stimulation; KHS, Krebs–Henseleit solution; L-NAME, Nω nitro-L-arginine methyl ester; nNOS, neuronal nitric oxide synthase; TTX, tetrodotoxin

INTRODUCTION

The combination of obesity and hypertension is associated with high morbidity and mortality because it leads to cardiovascular and kidney disease – effects seen in developed countries, as well as low-income populations [1]. Therefore, prevention of obesity becomes an important goal in overall efforts to control blood pressure and reduce the incidence of hypertension-related cardiovascular complications. Potential mechanisms linking obesity to hypertension include metabolic, endothelial and vascular dysfunction, neuroendocrine imbalances, sodium retention, glomerular hyperfiltration, proteinuria, and maladaptive immune and inflammatory responses [1–3]. However, the complex interactions between obesity and hypertension still raise many questions regarding the exact mechanisms involved and the proof of their causes and effects.

Arterial tone is regulated by the central nervous system, perivascular innervations, and myogenic mechanisms, as well as by endothelial and humoral factors. Perivascular innervation plays a principal role in the regulation of vascular tone, specifically in certain vessels such as the mesenteric vascular bed [4,5], where blood flow is approximately 20–30% of the total cardiac output [6]. Inadequate mesenteric blood flow and tissue perfusion can produce relevant hemodynamic changes [6–8]. This regulation involves sympathetic, nitrergic, and sensory innervations, which release mainly noradrenaline or ATP from sympathetic nerve terminals, nitric oxide from nitrergic innervations, and calcitonin gene-related peptide (CGRP) from sensory innervation [5,9–11]. Alterations in the functional

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role of these components have been associated with several pathophysiological circumstances [12–16].

The amount and the kind of dietary fatty acids can regulate complex intracellular signaling systems, thereby modulating cellular function [17]. In a previous study, we had demonstrated that neural control of mesenteric vasomotor tone is altered in rats fed a high-fat diet (HFD) for a short term, increasing the adrenergic and decreasing the nitrergic components, the effects of which are avoided by rosuvastatin treatment [18]. Additionally, we have recently demonstrated, in mesenteric arteries from hypertensive rats, that moderate aerobic exercise training decreased sympathetic activity and increased neuronal nitric oxide release and bioavailability [19].

Taking into account these results, we hypothesized that an aerobic training program might contribute to avoid the vascular effects of a HFD intake and consequently the development of obesity-linked hypertension, thus leading to a reduction in the administration of any pharmacological treatment. Thus, our objective was to study the effect of aerobic exercise training on sympathetic, nitrergic, and sensory innervation function in superior mesenteric artery from rats fed with HFD.

METHODS

Animals

Three-month-old male Wistar rats (initial weight $334.6 + 3.9$ g) were obtained from the Animal Quarters of the Universidad Autónoma de Madrid (Registration number EX-021U), in accordance with guidelines 609/86 of the E.E.C., R.D. 233/88 of the Ministerio de Agricultura, Pesca y Alimentación of Spain, and Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health (NIH publication No. 85.23 revised 1985). All experimental procedures involving animal use were approved by the Ethics Committee of the Universidad Autónoma de Madrid (RD 1201/2005).

Rats were housed at a constant room temperature, humidity and light cycle (12 : 12-h light–dark) and had free access to tap water and standard rat chow *ad libitum*. Animals were weighed every 2 weeks. Animals were divided in three experimental groups: control group, fed with a standard chow (3% fat); sedentary HFD rats, fed with HFD chow (35% fat) for 8 weeks; and trained HFD rats, fed with the HFD chow and simultaneously subjected to an aerobic exercise training protocol for 8 weeks. Diets were purchased from Panlab S.A. (Spain).

Exercise training protocol

Exercise training was performed on a motor treadmill (Motor-driven Treadmill LI8706; Letica Scientific Instruments, Barcelona, Spain) for 8 weeks, five times per week for 50 min each session, gradually progressing towards 55–65% of maximal running speed (15–20 m/min), as described elsewhere [19–21]. To determine the maximal exercise capacity, the rats were subjected to a progressive exercise test on a treadmill using an incremental speed protocol of 5 m/min every 3 min and no grade until exhaustion. The treadmill exercise test was repeated after 5 weeks of exercise training in order to adjust training intensity. Rats were considered to be exhausted when they could no longer run at the treadmill speed. The sedentary rats were handled at least twice a week for habituation to the experimental protocols.

Blood pressure measurements

At the end of the training protocol, and 24 h after the last exercise session, the mean blood pressure was measured using the tail-cuff method, as previously described [19].

Tissue samples

At the end of the training protocol, and 24 h after the last session of exercise training, the rats were euthanized by decapitation. The soleus muscle was removed and immediately stored at −80°C until citrate synthase analysis was performed. The superior mesenteric artery was carefully dissected, cleaned of connective tissue, and divided into 2-mm-long segments. Measurements were taken using a micrometer eyepiece mounted on a Euromex Holland binocular lens. Segments were maintained in cold (4°C) Krebs–Henseleit solution (KHS) (in mmol/l: 115 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4.7H_2O$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 11.1 glucose, and 0.01 Na_2 EDTA) bubbled with a 95% O_2–5% CO_2 mixture. Mesenteric adipose tissue was carefully dissected and wet weight was measured. For protein expression analysis, some segments were endothelium-denuded and quickly frozen in liquid nitrogen, being maintained at −80°C until the day the experiments were performed.

Some segments were mounted on 100-μm wires in a small vessel myograph to measure the internal diameter, as previously described [22]. The effective internal lumen diameter was $1.5 + 0.2$ mm. No differences were found among the experimental groups.

Citrate synthase activity

Citrate synthase activity was determined in mixed right soleus as previously reported [19,23] and used as a marker of muscle oxidative activity. The enzyme activity was measured in whole muscle homogenates, and the complex resulting from acetyl-CoA and oxaloacetate was determined at 412 nm and 25°C, at an interval of 10 min. Citrate synthase activity was expressed as nmol/min per mg of protein.

Vascular reactivity

The method used for isometric tension recording has been described in full elsewhere [11,24]. Two parallel stainless steel pins were introduced through the lumen of the vascular segment: one was fixed to the bath wall and the other connected to a force transducer (Grass FTO3C; Quincy, Massachusetts, USA); this, in turn, was connected to a model 7D Grass polygraph. For electrical field stimulation (EFS) experiments, segments were mounted between two platinum electrodes 0.5 cm apart and connected to a stimulator (Grass, model S44) modified to supply adequate current strength. Segments were suspended in an organ bath containing 5 ml of KHS at 37°C and continuously bubbled with a 95% O_2–5% CO_2 mixture (pH of 7.4). Some experiments were performed in endothelium-denuded segments to eliminate the main source of vasoactive substances, including endothelial nitric oxide. This avoided

possible actions by different drugs on endothelial cells that could lead to misinterpretation of results. Endothelium was removed by gently rubbing the luminal surface of the segments with a thin wooden stick. The segments were subjected to an optimal resting tension of 0.5 g, which was readjusted every 15 min during a 90-min equilibration period before drug administration. After this, the vessels were exposed to 75 mmol/l KCl to check their functional integrity. Endothelium removal did not alter the contractions elicited by KCl. After a washout period, the presence/absence of vascular endothelium was tested by the ability of 10 μmol/l acetylcholine (ACh) to relax segments precontracted with noradrenaline. Arteries which relaxed more than 90% or less than 10% of the previous tone obtained by noradrenaline were respectively considered as endothelium-intact or endothelium-denuded.

Frequency–response curves to EFS were performed. The parameters used for EFS were 200 mA, 0.3 ms, at frequencies ascending from 1 to 16 Hz, for 30 s each frequency, with an interval of 1 min between each stimulus, the time required to recover basal tone. A washout period of at least 1 h was necessary to avoid desensitization between consecutive curves. Two successive frequency–response curves separated by 1-h intervals produced similar contractile responses. To evaluate the neural origin of the EFS-induced contractile response, the nerve impulse propagation blocker, tetrodotoxin (TTX, 0.1 μmol/l), was added to the bath 30 min in advance.

To determine the participation of sympathetic innervation in the EFS-induced response in segments from all experimental groups, 1 μmol/l phentolamine, an α-adrenoceptor antagonist, 0.1 mmol/l suramin, a nonspecific P2 purinergic receptor antagonist, or a combination of phentolamine + suramin, was added to the bath 30 min before performing the frequency–response curve. Additionally, the vasoconstrictor response of exogenous noradrenaline (1 nmol/l–10 μmol/l) was tested in segments from both the experimental groups.

To analyze the participation of nitric oxide in the EFS-induced response in segments from both groups of rats, 0.1 mmol/l N^{ω}-nitro-L-arginine methyl ester (L-NAME) – a nonspecific inhibitor of nitric oxide synthase (NOS) – was added to the bath 30 min before performing the second frequency–response curve. The vasodilator response to the nitric oxide donor – diethylamine NONOate (DEA-NO, 0.1 nmol/l–0.1 mmol/l) – was determined in noradrenaline-precontracted arteries from both the experimental groups.

To study the possible participation of sensory innervation in the EFS-induced response, 0.5 μmol/l CGRP (8–37) – a CGRP receptor antagonist – was added to the bath 30 min before performing the second frequency–response curve.

Noradrenaline and ATP levels

Noradrenaline and ATP releases were measured using Noradrenaline Research EIA (Labor Diagnostica Nord, Gmbh and Co., KG, Nordhon, Germany) or an ATP Colorimetric/Fluorometric Assay kit (Abcam, Cambridge, UK), following the manufacturers' instructions. Endothelium-denuded segments of rat mesenteric arteries from all experimental groups were preincubated for 30 min in 5 ml of KHS at 37° C and continuously gassed with a 95% O_2–5% CO_2 mixture (stabilization period). This was followed by two washout periods of 10 min in a bath of 0.4 ml of KHS. Then, the medium was collected to measure basal release. Afterwards, the organ bath was refilled, and cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz were applied at 1-min intervals. Afterwards, the medium was collected to measure EFS-induced neurotransmitter release. Samples were immediately frozen in liquid nitrogen and conserved at −70°C until experiments were performed. Results were expressed as ng noradrenaline/ml mg tissue or nmol ATP/ml mg tissue.

Nitric oxide measurements

Nitric oxide release was measured using fluorescence emitted by the fluorescent probe 4,5-diaminofluorescein (DAF-2), as previously described [18,19,25]. Endothelium-denuded mesenteric arteries from all experimental groups were subjected to a 60-min equilibration period in (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (in mmol/l: NaCl 119; HEPES 20; $CaCl_2$ 1.2; KCl 4.6; $MgSO_4$ 1; KH_2PO_4 0.4; $NaHCO_3$ 5; glucose 5.5; Na_2HPO_4 0.15; pH 7.4) at 37° C. Arteries were incubated with 2 μmol/l DAF-2 for 30 min. The medium was then collected to measure basal nitric oxide release. Once the organ bath was refilled, cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz at 1-min intervals were applied. Afterwards, the medium was collected to measure EFS-induced nitric oxide release. The fluorescence of the medium was measured at room temperature using a spectrofluorometer (LS50 Perkin Elmer Instruments, FL WINLAB Software, Waltham, Massachusetts, USA) with excitation wavelength set at 492 nm and emission wavelength at 515 nm.

The EFS-induced nitric oxide release was calculated by subtracting basal nitric oxide release from that evoked by EFS. Also, blank sample measures were collected in the same way from segment-free medium in order to subtract background emission. Some assays were performed in the presence of either TTX (0.1 μmol/l) or the specific nNOS inhibitor 7-NI (0.1 mmol/l). The amount of nitric oxide released was expressed as arbitrary units/mg tissue.

Superoxide anions measurements

Superoxide anion levels were measured using lucigenin chemiluminescence, as previously described [19,25]. Endothelium-denuded mesenteric segments from all groups of rats were rinsed in KHS for 30 min, equilibrated for 30 min in HEPES buffer at 37°C, transferred to test tubes that contained 1 ml HEPES buffer (pH 7.4) with lucigenin (5 μmol/l), and then kept at 37°C. The luminometer was set to report arbitrary units of emitted light; repeated measurements were collected for 5 min at 10-s intervals and averaged. 4,5-dihydroxy-1,3-benzene-disulphonic acid 'Tiron' (10 mmol/l) – a cell-permeant, nonenzymatic superoxide anion scavenger – was added to quench the superoxide anion-dependent chemiluminescence. Calculations were performed subtracting the lucigenin chemiluminescence obtained in the presence of Tiron from that obtained in its absence. Blank measurements were performed in the

same way without mesenteric segments to subtract background emission.

Western blot analysis

Western blot analysis was performed as previously described [18,25]. For these experiments, we used rabbit polyclonal antibodies against nNOS (1 : 2000; Abcam), nNOS phosphorylated in Serine 1177 (P-nNOS, 1 : 2000; Abcam), a mouse monoclonal antibody against tyrosine hydroxylase (1 : 500; Santa Cruz Biotechnology, Texas, USA), and monoclonal anti-β-actin-peroxidase antibody (1 : 50 000; Sigma-Aldrich, Madrid, Spain). Rat brain homogenates were used as positive control.

Drugs used

Acetylcholine chloride, CGRP (8–37), DAF-2, DEA-NO, diethylammonium salt, L-NA hydrochloride, L-NAME hydrochloride, suramin, 7NI, phentolamine, lucigenin, Tiron, and TTX (Sigma-Aldrich) were used. Stock solutions (10 mmol/l) of drugs were made in distilled water, except for noradrenaline, which was dissolved in a NaCl (0.9%)–ascorbic acid (0.01% w/v) solution. These solutions were kept at −20°C, and appropriate dilutions were made in KHS on the day of the experiment.

Data analysis

The responses elicited by EFS and noradrenaline were expressed as a percentage of the initial contraction elicited by 75 mmol/l KCl for comparison between experimental groups. The relaxation induced by DEA-NO was expressed as a percentage of the initial contraction elicited by noradrenaline (control: 1134.6 + 5.6 mg; sedentary HFD: 1243.2 + 7.5 mg; trained HFD: 1254.9 + 9.5 mg; $P > 0.05$). Results are given as mean + SEM. Statistical analysis was performed by comparing the curve obtained in the presence of the different substances with the previous or control curve by means of two-way analysis of variance (ANOVA), using GraphPad Prism 5.0 software (California, USA). For nitric oxide, superoxide anion, ATP, and noradrenaline release data, the statistical analysis was done using one-way ANOVA followed by the Newman–Keuls post-hoc test. P less than 0.05 was considered significant.

RESULTS

Animal evolution

We weighed the animals every 2 weeks, observing a significantly higher increase in HFD rats compared to control rats. The increase in body weight was higher in sedentary compared to trained HFD animals. When measuring food intake, it was lower in both sedentary and trained HFD rats compared to controls throughout the experiment, whereas calorie intake was higher in sedentary and trained HFD rats than in controls. Mean blood pressure was similar in all experimental groups. After sacrifice, we observed that mesenteric adipose tissue wet weight was higher in sedentary HFD rats than in control and trained HFD rats. Additionally, citrate synthase activity in mixed soleus was significantly higher in trained HFD compared to control and sedentary HFD animals (Table 1).

Vasomotor response to potassium chloride

In endothelium-intact mesenteric segments, the vasoconstrictor response to 75 mmol/l KCl was similar in all experimental groups (control: 1772 ± 129 mg; sedentary HFD: 1797 ± 121 mg; trained HFD: 1699 ± 112 mg; $P > 0.05$; $n = 12–16$ animals each group). Endothelium removal did not alter KCl-induced vasoconstriction (control: 1848 ± 81 mg; sedentary HFD: 1968 ± 100 mg; trained HFD: 1852 ± 80 mg; $P > 0.05$; $n = 12–16$ animals each group).

Vasodilator response to acetylcholine

Acetylcholine induced cumulative concentration and endothelium-dependent relaxations in noradrenaline-contracted arteries from all experimental groups. This vasodilation was lower in sedentary HFD segments compared to control or to trained HFD animals (Fig. 1a).

Vascular responses to electrical field stimulation

The application of EFS induced a frequency-dependent contractile response in endothelium-intact mesenteric segments from all experimental groups. This vasoconstriction was greater in segments from sedentary HFD rats compared to control and trained HFD rats (Fig. 1b). Endothelium removal increased EFS-induced contractile response similarly in segments from all experimental groups (Fig. 1c and d). EFS-induced contractions were practically abolished in segments from all experimental groups by the neurotoxin TTX (0.1 mmol/l), indicating the neuronal origin of the factors inducing this response ($n = 10–12$ animals each group; results not shown).

TABLE 1. Animal evolution

	Control	Sedentary HFD	Trained HFD
Body weight gain (g)	88.1 ± 8.4	164.2 ± 9.8*	125.6 ± 7.4**
Mesenteric adipose tissue (g/cm tibia)	0.97 + 0.04	2.04 + 0.12*	1.62 + 0.09**
Food intake (g/day)	21.6 ± 0.2	18.5 ± 0.4*	16.2 ± 0.3*
Food intake (kcal/day)	64.8 ± 0.6	99.9 ± 2.2*	87.5 ± 1.6*
Mean blood pressure (mmHg)	123.2 + 3.3	122.5 + 3.0	122.9 + 2.2
Citrate synthase activity (nmol/min/mg protein)	59.5 ± 3.9	48.9 ± 1.0*	69.0 ± 4.2**

Mesenteric adipose tissue is expressed per tibia length. Food intake was measured every day. All data are expressed as mean + SEM. $n = 12–16$ rats in each group.
HFD, high-fat diet.
*$P < 0.05$ vs. control.
**$P < 0.05$ vs. sedentary HFD.

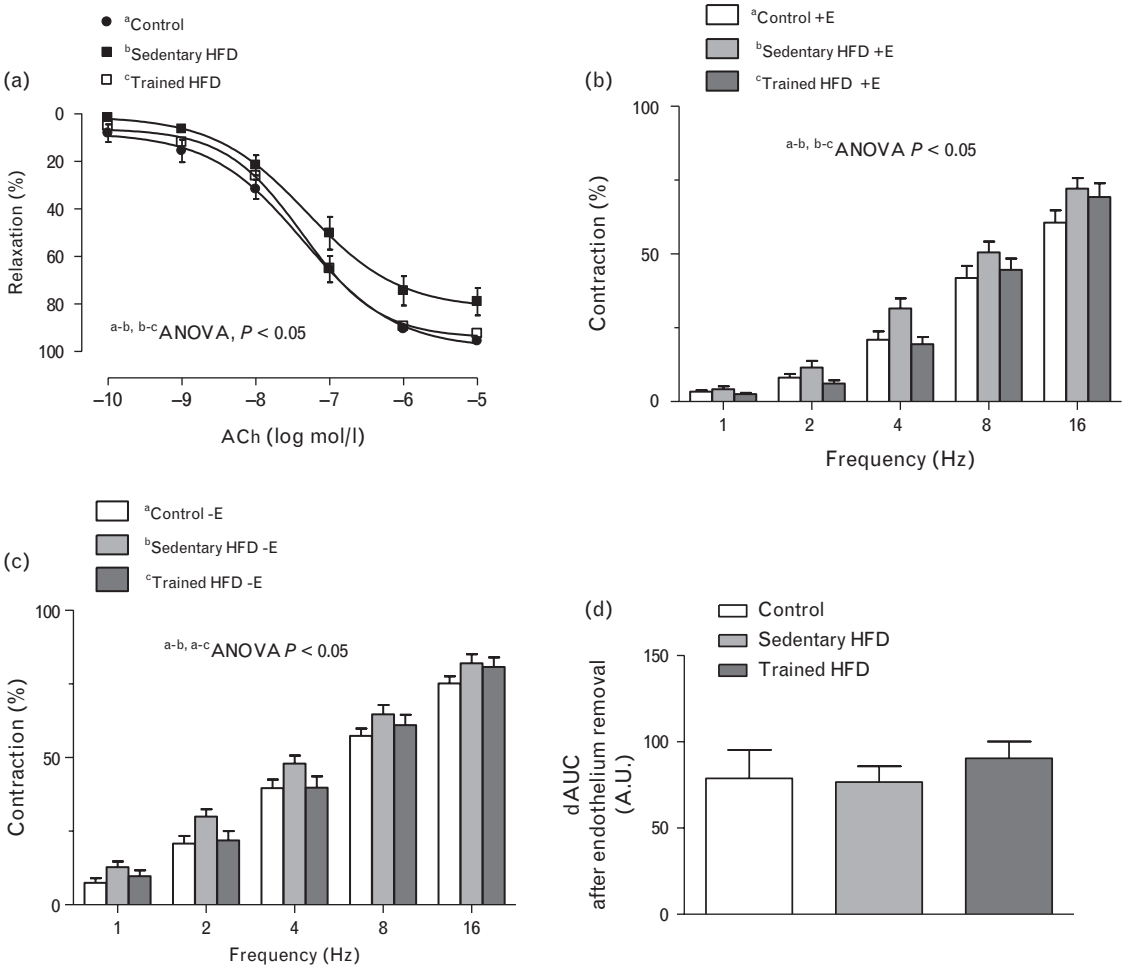

FIGURE 1 Vasodilator response to acetylcholine and vasoconstrictor response to electrical field stimulation. ACh-induced vasodilation in endothelium-intact mesenteric segments from control, sedentary and trained HFD rats (a). Results (mean ± SEM) were expressed as a percentage of the previous tone elicited by exogenous NA ($n = 8–10$ animals in each group). EFS-induced vasoconstriction in endothelium intact (b) and endothelium denuded (c) mesenteric segments from control, sedentary, and trained HFD rats. Results (mean ± SEM) were expressed as a percentage of the initial contraction elicited by KCl ($n = 12–16$ animals in each group). Results (mean ± SEM) were expressed as a percentage of the initial contraction elicited by KCl. (d) Differences of area under the curve (dAUC) in the presence or absence of endothelium. ACh, acetylcholine; EFS, electric field stimulation; HFD, high-fat diet; NA, noradrenaline.

Participation of the sympathetic component of mesenteric vascular innervation

Preincubation with the α-adrenergic antagonist phentolamine (1 μmol/l) decreased the vasoconstrictor response induced by EFS in endothelium-denuded segments from all experimental groups (Fig. 2a–c). This decrease was greater in mesenteric segments from sedentary HFD animals (Fig. 3d). There was a remnant phentolamine-resistant contractile response, which was lower in mesenteric segments from sedentary HFD (Fig. 2a–c). Preincubation with the nonspecific P2 purinergic receptor antagonist suramin (0.1 mmol/l) decreased EFS-induced vasoconstriction in segments from control and trained HFD rats, but did not modify it in artery segments from sedentary HFD rats (Fig. 3a–c). Combined preincubation with phentolamine + suramin decreased EFS-induced vasoconstriction in segments from control and trained HFD animals compared to segments preincubated only with phentolamine, whereas it did not modify it in mesenteric rings from sedentary HFD (Fig. 2a–c).

Noradrenaline-induced vasoconstriction was similar in all experimental groups (Fig. 3a). Tyrosine hydroxylase expression was similar in all experimental groups (Fig. 3b). EFS-induced noradrenaline release was higher in mesenteric segments from sedentary HFD compared to control and trained HFD (Fig. 3c). EFS-induced ATP release was lower in mesenteric segments from sedentary HFD compared to segments from control or trained HFD animals (Fig. 3d).

Participation of the nitrergic component of mesenteric vascular innervation

Preincubation with the unspecific NOS inhibitor L-NAME (0.1 mmol/l) significantly increased the EFS-contractile response in endothelium-denuded segments from control and trained HFD rats, but did not modify EFS-induced vasoconstriction in arteries from sedentary HFD animals (Fig. 4). EFS induced nitric oxide release in segments from all groups. This release was lower in sedentary HFD mesenteric segments (Fig. 5a). 7NI practically abolished EFS-induced nitric oxide release ($n = 8–10$ animals each group; results not shown). Both nNOS and P-nNOS expressions were lower in homogenates from sedentary HFD arteries

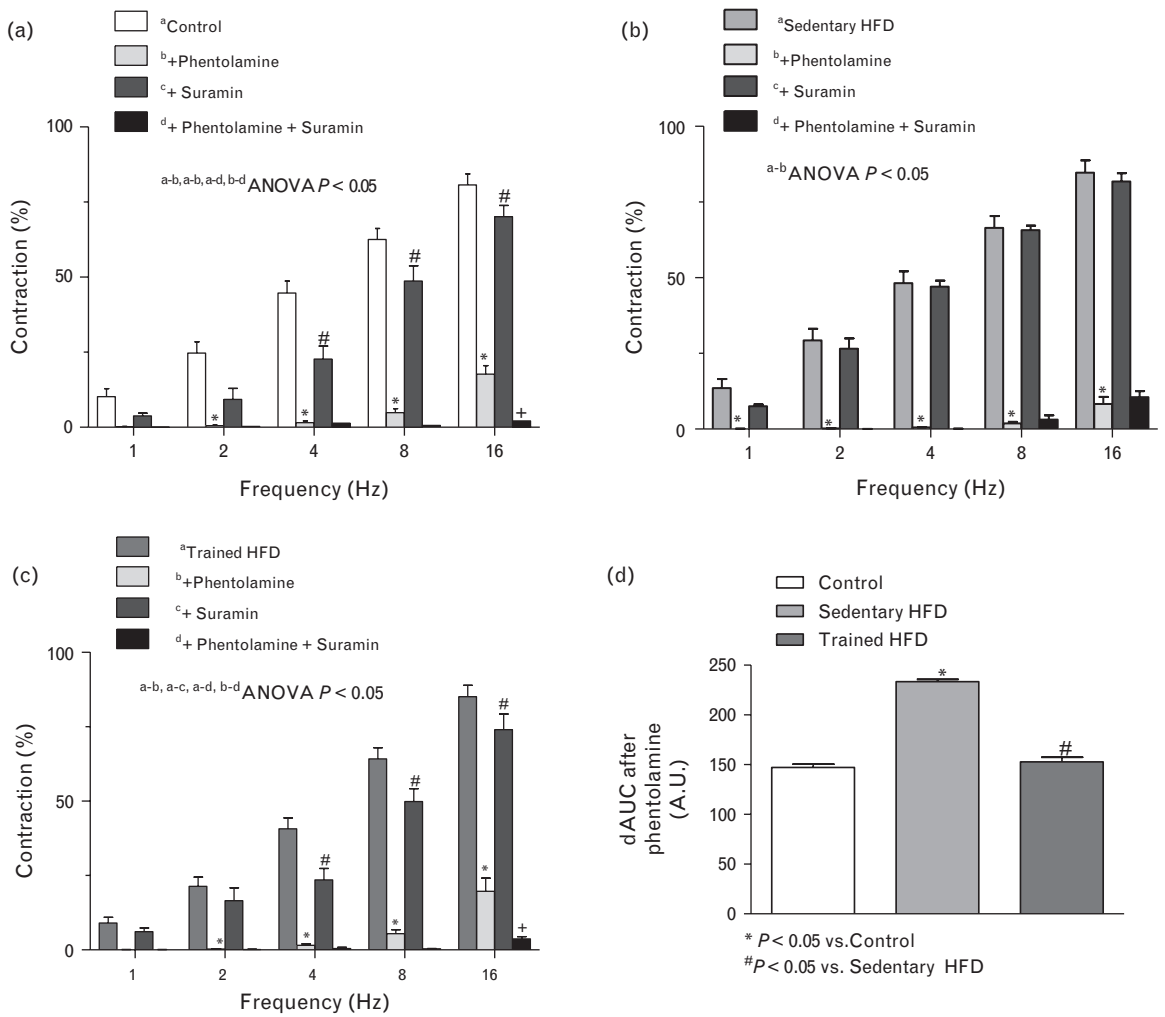

FIGURE 2 Effect of obesity and aerobic training on sympathetic innervation function. Effect of preincubation with 1 μmol/l phentolamine, 0.1 mmol/l suramin, or a combination of phentolamine + suramin on the vasoconstriction response induced by EFS in endothelium-denuded mesenteric segments from control (a) sedentary HFD (b) and trained HFD animals (c). Results (mean ± SEM) were expressed as a percentage of the initial contraction elicited by KCl ($n = 8–12$ animals in each group). (d) Differences of area under the curve (dAUC) in the absence or presence of 1 μmol/l phentolamine. dAUC values are expressed as arbitrary units. ACh, acetylcholine; EFS, electric field stimulation; HFD, high-fat diet.

compared with control and trained HFD segment homogenates (Fig. 5b). Neither the vasodilator response to DEA-NO nor the superoxide anion production were modified in our experimental conditions (Fig. 5c and d).

Participation of the sensory component in vascular responses to electrical field stimulation

Preincubation with the CGRP receptor antagonist CGRP (8–37) (0.5 μmol/l) did not alter the EFS-induced contraction in any experimental group (Fig. 6).

DISCUSSION

In this study, we observed that moderate aerobic exercise averts the alterations in perivascular innervation function induced by HFD administration, since we observed that the vasoconstrictor response to EFS and the function of sympathetic and nitrergic innervation in mesenteric arteries from trained HFD rats remained unmodified compared to the control group.

We have previously reported that short-term administration of a HFD diet induced an increased response to EFS in mesenteric arteries, with the net effect being an: adrenergic hyperactivity associated with an increased release and vasoconstrictor response to noradrenaline; decreased ATP release; and diminished neuronal nitric oxide release. Consequently, HFD produces an increase of peripheral vascular resistance [18]. Since we have previously reported that aerobic exercise training decreased EFS-induced vasoconstriction in mesenteric artery from spontaneously hypertensive rats, in the present study, we analyzed the possibility that moderate exercise could avert the neuronal dysfunction observed in HFD rats.

In the three experimental groups, EFS produced a frequency-dependent contraction that was abolished in the presence of TTX, indicating that the vasomotor responses were the result of neurotransmitter release from perivascular nerves. HFD produced an increased response to EFS, which was not observed in trained rats. This effect was not attributable to changes in the intrinsic contractile machinery, as demonstrated by the similar vasoconstrictor

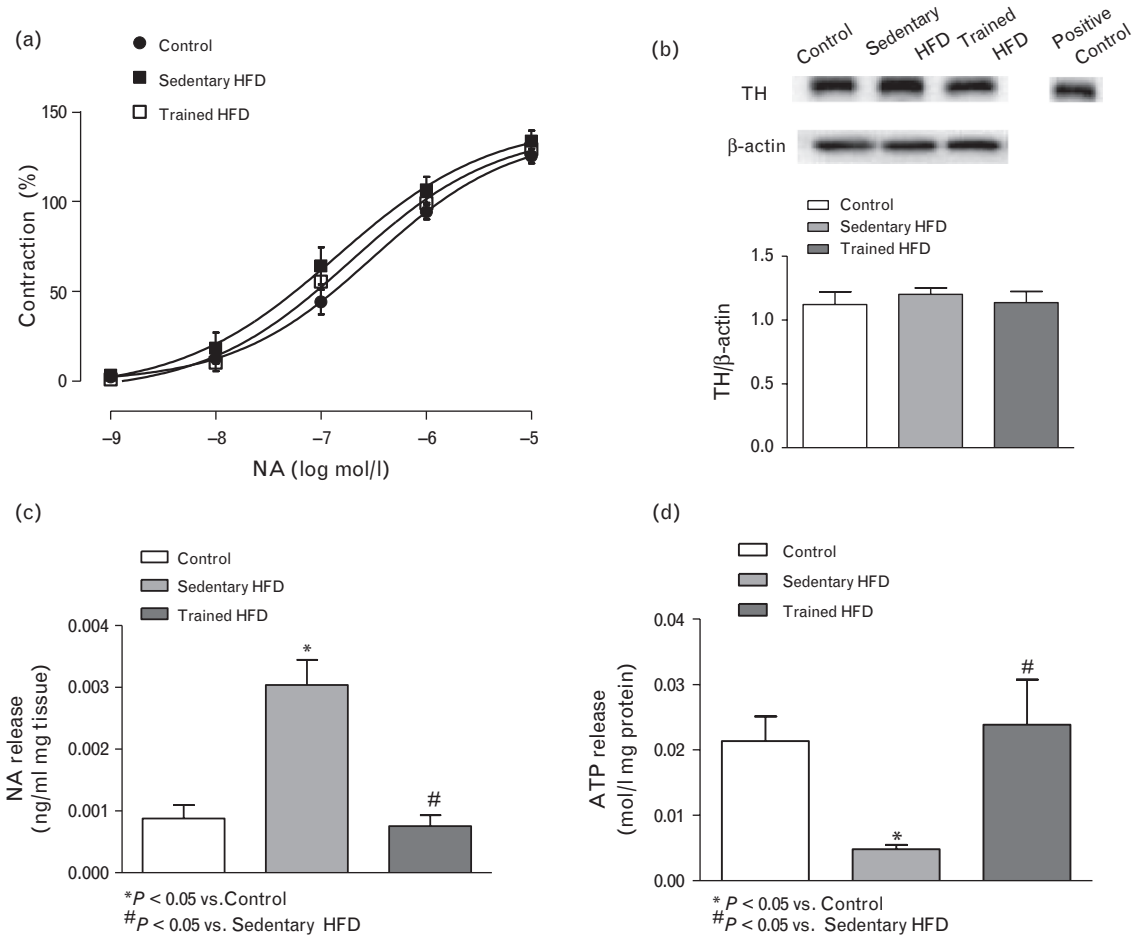

FIGURE 3 Effect of obesity and aerobic training on NA synthesis and vasoconstriction, and ATP release (a) vasoconstrictor response to NA in segments from control, sedentary, and trained HFD rats. Results (mean ± SEM) were expressed as a percentage of the initial contraction elicited by KCl ($n = 8–10$ animals in each group). (b) TH expression in endothelium-denuded mesenteric segments from control, sedentary and trained HFD rats. The blots are representative of eight separate segments from each group. Rat brain homogenates were used as a positive control. Lower panel shows relation between TH expression and β-actin. Results (mean ± SEM) are expressed as a ratio of the signal obtained for each protein and the signal obtained for β-actin. EFS-induced NA (c) and ATP (d) releases in mesenteric segments from control, sedentary, and trained HFD rats. Results (mean + SEM) were expressed as ng NA/ml mg tissue or nmol ATP/ml mg tissue ($n = 8$ animals per group). EFS, electric field stimulation; HFD, high-fat diet; NA, noradrenaline; TH, tyrosine hydroxylase.

response to KCl in the three experimental groups. The reported discrepancies regarding KCl response [18,19,26–32] could be attributed to differences in the vascular bed analyzed and/or the kind of experimental models of obesity and training used.

Endothelial factors have been described to modulate the response to several vasomotor substances, including neurotransmitters [9,33]. Additionally, obesity impairs endothelial function [1,3,34,35], whereas aerobic exercise training improves or attenuates the progression of endothelial dysfunction observed in obesity [36–40]. Consequently, the response to EFS could be differentially affected by the endothelium in the different experimental groups. Our next objective was to analyze the response to ACh. As has been reported, endothelial function was impaired in mesenteric segments from sedentary HFD rats, and this did not occur in segments from trained HFD animals. However, endothelium removal increased vasoconstrictor response to EFS to the same extent in the three experimental groups. Thus, despite the observed differences in endothelial function, the modulating role of endothelium on EFS response is not modified in our experimental conditions. So, we can conclude that the differences observed in EFS-induced vasoconstriction are only innervation-dependent. For that reason, we performed the following experiments in endothelium-removed mesenteric segments.

Obesity is usually accompanied by increased sympathetic discharge [18,41–44]. In line with this, several studies have demonstrated an elevation in renal sympathetic nerve activity due to adipokine disorders, such as leptin, adiponectin, and resistin-activation of the renin–angiotensin system [45–48]. Additionally, aerobic exercise training is associated with reductions in sympathetic activity [19,49]. These evidences, together with the increased mesenteric adipose tissue observed in sedentary HFD rats compared to controls, which was lower in trained HFD rats, led us to explore the possible changes in sympathetic innervation function induced by exercise in sedentary HFD rats. The reduction of EFS-induced contractile response in the presence of alpha adrenergic antagonist phentolamine was higher in sedentary HFD rats and similar in trained HFD animals compared to controls, confirming that aerobic exercise training averts the increased activity by the adrenergic component of sympathetic innervation in arteries

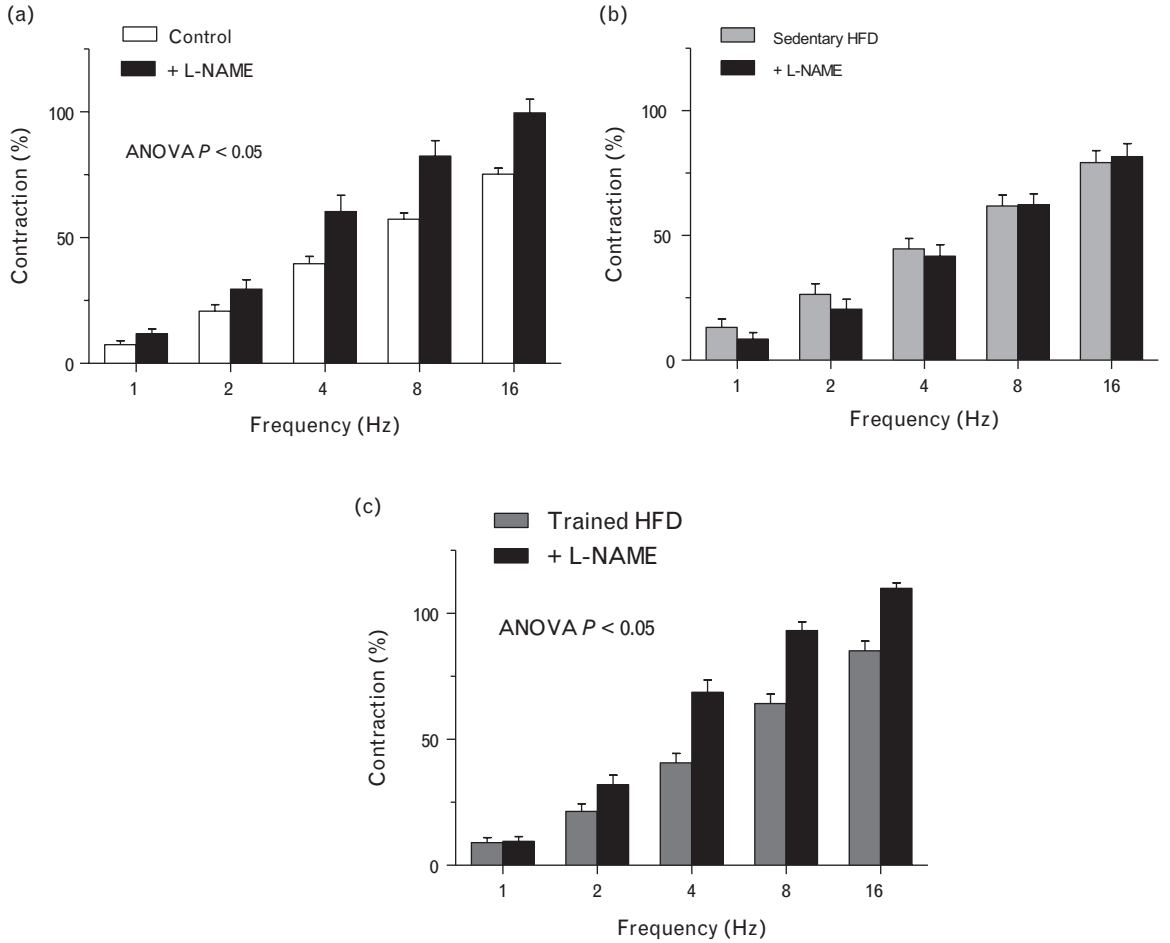

FIGURE 4 Effect of obesity and aerobic training on nitrergic innervation function. Effect of preincubation with 0.1 mmol/l L-NAME on the vasoconstrictor response induced by EFS in mesenteric segments from control (a), sedentary (b), and trained (c) HFD rats. Results (mean ± SEM) are expressed as a percentage of the previous contraction elicited by KCl ($n = 10–12$ animals in each group). EFS, electric field stimulation; HFD, high-fat diet; L-NAME, N^{ω} nitro-L-arginine methyl ester.

from obese rats [19,49–53]. Vasoconstrictor response to noradrenaline remained unmodified in all experimental groups This result contrasts to those observed in spontaneously hypertensive rats (SHR) mesenteric segments, where we observed an increase in noradrenaline vasoconstriction [19], suggesting that the effect of training is dependent on the basal situation of the experimental participant. Additionally, noradrenaline release was increased in mesenteric rings from sedentary HFD compared to segments from control and trained HFD rats. An increase in the activation of noradrenergic neurons resulting from a HFD intake has been described [54], as well as differential DNA methylation within the promoter regions of tyrosine hydroxylase [55]. However, to the best of our knowledge, there are no studies analyzing possible modifications in the noradrenaline synthesis pathway in peripheral nervous system. Thus, we analyzed tyrosine hydroxylase expression in our experimental conditions, observing a similar expression of this enzyme in all experimental groups. Thus, the increased noradrenaline release observed in arteries from obese rats might be due to an increase in tyrosine hydroxylase activity, and aerobic exercise training could avert this activation. Taken together, these results indicate that aerobic exercise training avoids the alterations in the adrenergic component of sympathetic innervation observed in HFD-induced obesity.

There was a remaining contractile response that was phentolamine-resistant in all experimental groups and was lower in sedentary HFD rats compared to control and trained HFD rats. We have reported that the remnant vasoconstriction observed after preincubation with phentolamine in segments from control rats is due to ATP release from sympathetic innervation [18,56]. In our experimental conditions, we observed a decreased EFS-induced vasoconstriction only in mesenteric segments from control and trained HFD animals after preincubation with suramin, whereas vasoconstriction was not modified in segments from sedentary HFD rats. In parallel, the results on ATP release confirm that HFD feeding reduces this release and training blocks this reduction. These results contrast with a previous study by Haddock and Hill [44], which reported an increased participation of purinergic neurotransmission in resistance arteries from HFD animals. The differences in the rat strain, the diet composition and time of administration, the vascular bed analyzed, and the experimental approach could explain these differences. Altogether, these results confirm that the HFD diet increased noradrenaline release and decreased ATP release, and training avoided these modifications.

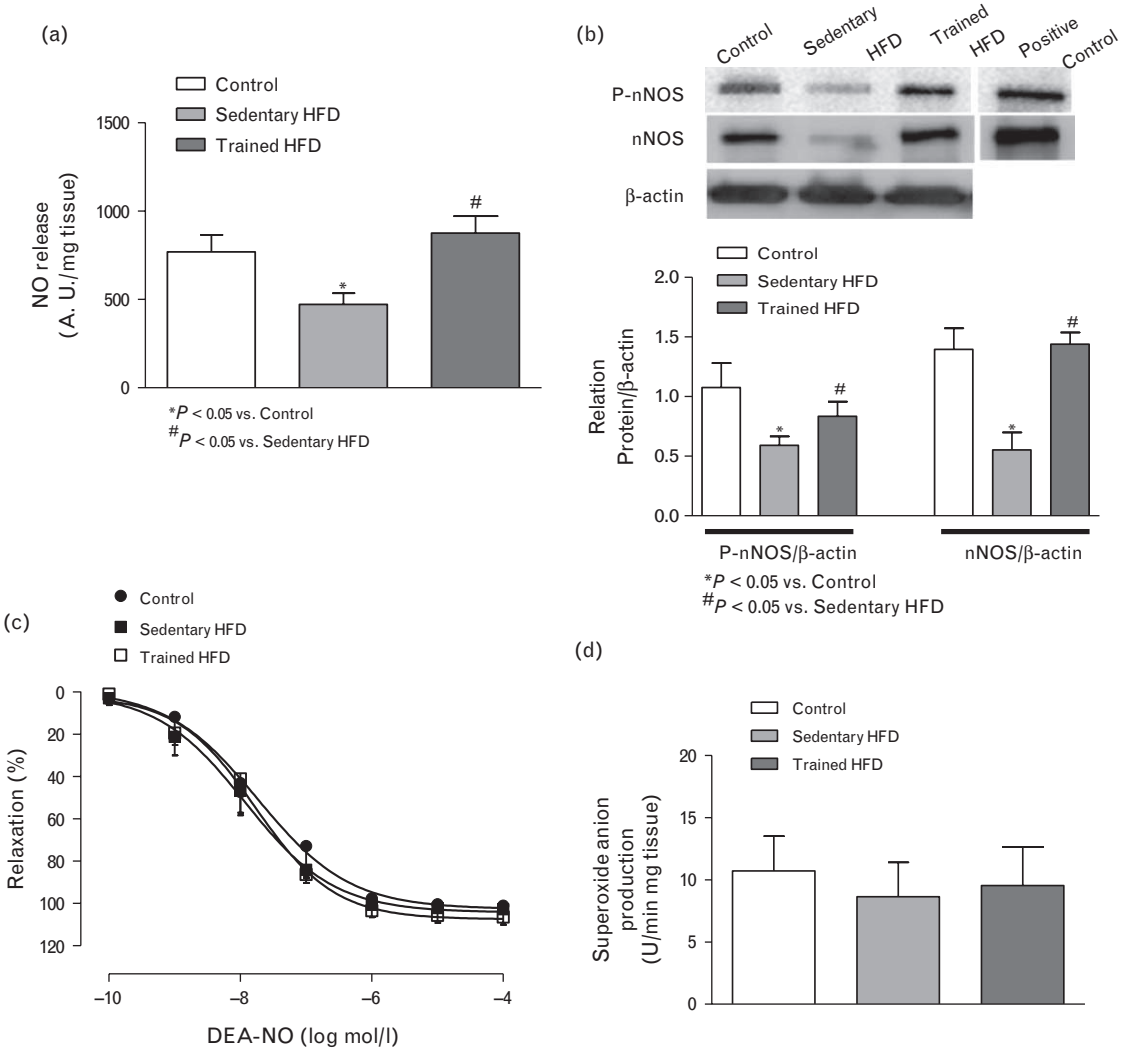

FIGURE 5 Effect of obesity and aerobic training on neuronal NO synthesis and vasodilation. (a) EFS-induced NO release in segments from control, sedentary, and trained HFD rats. Results (mean ± SEM) were expressed as arbitrary (AU)/mg tissue ($n = 6–8$ animals per group). (b) P-nNOS and nNOS expressions in endothelium-denuded mesenteric segments from control, sedentary, and trained HFD rats. The blots are representative of eight separate segments from each group. Rat brain homogenates were used as a positive control. Lower panel shows relation between protein expression and β-actin. Results (mean ± SEM) are expressed as ratio of the signal obtained for each protein and the signal obtained for β-actin. (c) Vasodilator response to NO donor DEA-NO in segments from control, sedentary and trained HFD rats. Results (mean ± SEM) are expressed as a percentage of the previous tone elicited by exogenous NA ($n = 8–10$ animals in each group). (d) Superoxide anion release in mesenteric segments from control, sedentary and trained HFD rats. Results (mean ± SEM) are expressed as chemiluminiscence units (u)/min mg tissue ($n = 6–8$ animals in each group). DEA-NO, diethylamine NONOate; EFS, electric field stimulation; HFD, high-fat diet; NA, noradrenaline; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; P-nNOS phosphorylated neuronal nitric oxide synthase.

Previously, we have demonstrated that the decreased release of nitric oxide from perivascular nitrergic innervation in segments from HFD rats contributed to the increased vasoconstrictor response to EFS [18]. Our next objective was to analyze whether, similarly to observations in sympathetic innervation, moderate exercise training averted the nitrergic dysfunction induced by a HFD diet. The nonspecific nitric oxide synthesis inhibitor L-NAME increased EFS vasoconstriction similarly in segments from control and trained HFD rats, but this increase was not present in segments from sedentary HFD animals, as previously reported [18]. These results indicate that aerobic exercise training averts the nitrergic innervation dysfunction observed in superior mesenteric artery from HFD-induced obese rats. The differences observed among our experimental groups are possibly related to modifications in nitric oxide release and/or vasodilator response. Nitric oxide release was lower in sedentary HFD segments than in either control or trained HFD rats, agreeing with the vascular reactivity studies. The fact that the specific nNOS inhibitor 7NI practically abolished nitric oxide release in the three experimental groups confirms, as we have demonstrated in this vascular bed, that this isoform is responsible for neuronal nitric oxide synthesis [18,19,24]. Additionally, the decreased nNOS and P-nNOS expressions observed in sedentary HFD compared to control and trained HFD rats correlate with the observed nitric oxide release. In agreement with these results, a decrease in nNOS expression due to HFD intake has been described in this vascular bed, and increases in nNOS through training have been reported in the central and peripheral nervous system and skeletal muscle [19,57–59].

Obesity has widely been described to be associated with an increase in oxidative stress [60], and moderate exercise

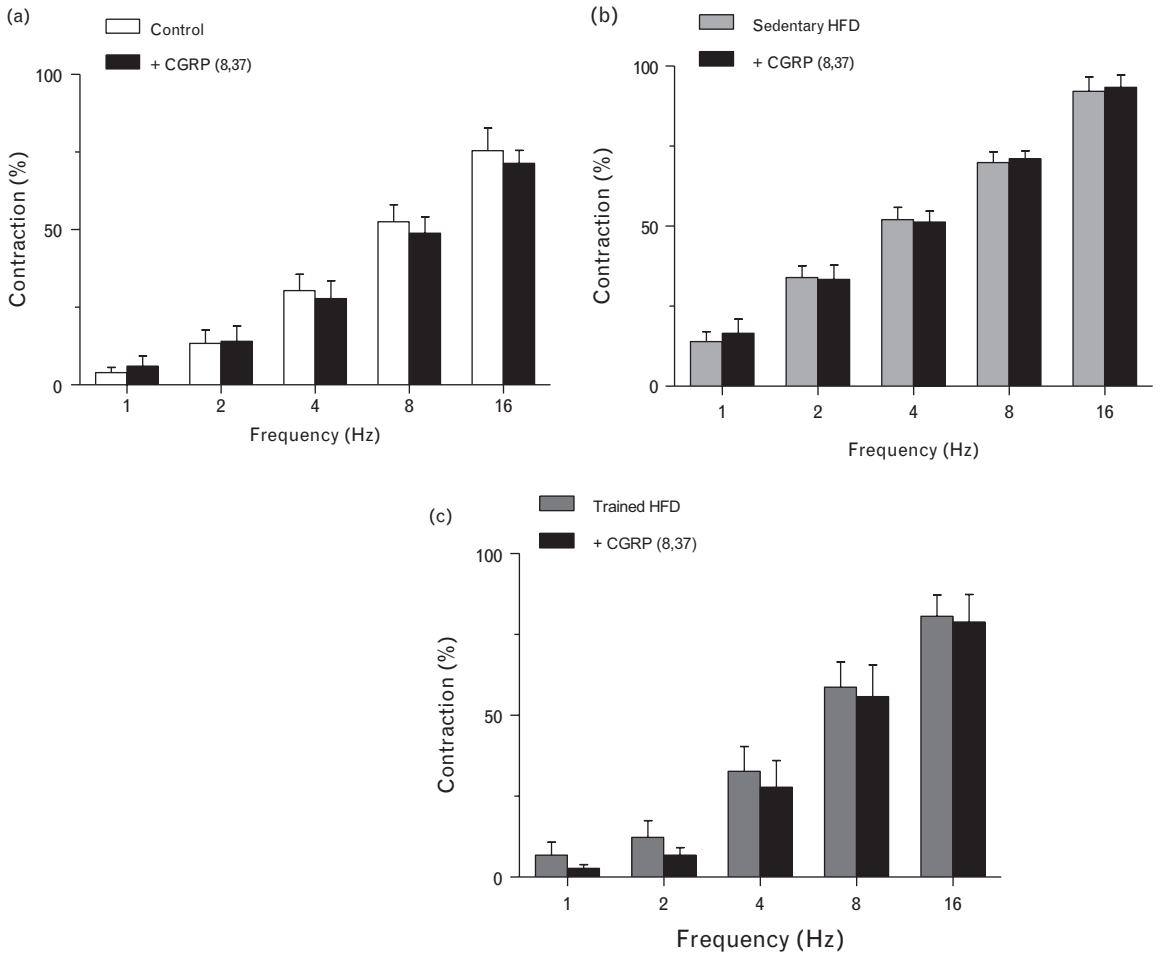

FIGURE 6 Effect of obesity and aerobic training on sensory innervation function. Effect of preincubation with 0.5 μmol/l CGRP (8–37) on the vasoconstrictor response induced by EFS in mesenteric segments from control (a), sedentary (b), and trained (c) HFD rats. Results (mean ± SEM) are expressed as a percentage of the previous contraction elicited by KCl ($n = 6–8$ animals in each group). CGRP, calcitonin gene-related peptide; EFS, electric field stimulation; HFD, high-fat diet.

training improves the vascular antioxidant defence systems, thus increasing nitric oxide bioavailability [19,61–63]. In our experimental conditions, superoxide anion production and the vasodilator response to DEA-NO were similar in segments from all experimental groups, suggesting that oxidative stress is not modified in animals fed HFD and/or subjected to moderate exercise. This result contrasts to observations in SHR, where the same training model decreased the superoxide anions [19], indicating that the antioxidant effect is associated not only to the training program, but also to the initial conditions of the study participant. Taken together, these results indicate that moderate exercise training averts the nitrergic dysfunction observed in HFD-induced obese rats by avoiding the described reduction in neuronal nitric oxide release.

We have previously reported that CGRP released from sensory innervation of this artery does not participate in the vasoconstrictor response to EFS in a healthy or obese situation [18,25,56,64]. On the contrary, aerobic exercise training has been shown to modulate the role of sensory innervation in several tissues including vascular tissue [19,65]. However, preincubation with the CGRP receptor antagonist CGRP (8–37) did not modify EFS-induced vasoconstriction in any group, indicating that sensory innervation is not altered in our experimental conditions.

The results regarding modifications in blood pressure following a HFD intake are controversial; both increases and no modifications in blood pressure have been reported depending on the diet fat composition and the time the diet has been given to the animals [66–71]. When measuring mean blood pressure, we observed no increase in HFD animals. However, the modifications we described could increase peripheral vascular resistance, which would participate in the future genesis of obesity-related hypertension. Additionally, aerobic exercise training did not modify blood pressure in obese animals, as reported in normotensive animals [72,73], but it did avoid the enhanced sympathetic and diminished nitrergic function observed in HFD-induced obesity. Altogether, these results suggest that aerobic exercise could reduce peripheral vascular resistance in obesity, thereby contributing to avoiding the future development of obesity-related hypertension.

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Conflicts of interest

There are no conflicts of interest.

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Reviewers' Summary Evaluations

Referee 1

Strengths: 1. The work addresses a clinically important issue, i.e. sympathetic regulation of the vasculature in obesity. 2. The focus on adrenergic and purinergic neurotransmission is novel. 3. The experiments were appropriately designed, analyzed and interpreted.

Weaknesses: 1. The observed changes in neurotransmission were small in magnitude and thus of questionable physiological relevance. 2. The data conflict in some important ways with the only other published study on the topic.

Referee 2

This paper demonstrates in adult rats that several in-vitro measures of sympathetic neural function of superior mesenteric arteries were enhanced by a high-fat diet. These increases in neural sympathetic function were inhibited if rats were exercised. Conversely, neural nitrergic function was decreased by high-fat diet, but restored by exercise. Surprisingly, no differences in tail-cuff measured blood pressure were observed between the groups. In future, 24-h telemetric measurement of blood pressure and heart rate (or better cardiac output) would be useful to test the authors' conclusion that the high-fat diet may cause increased vascular resistance, and that this is ameliorated by exercise.

Artículo 7: Effects of lipopolysaccharide on the neuronal control of mesenteric vascular tone in rats: mechanisms involved.

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EFFECTS OF LIPOPOLYSACCHARIDE ON THE NEURONAL CONTROL OF MESENTERIC VASCULAR TONE IN RATS: MECHANISMS INVOLVED

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ABSTRACT—The aim of the present study was to investigate the effects of lipopolysaccharide (LPS) on the contractile response induced by electrical field stimulation (EFS) in rat mesenteric segments, as well as the mechanisms involved. Effects of LPS incubation for 2 or 5 h were studied in mesenteric segments from male Wistar rats. Vasomotor responses to EFS, nitric oxide (NO) donor DEA-NO, and noradrenaline (NA) were studied. Phosphorylated neuronal NO synthase protein expression was analyzed, and NO, superoxide anion ($O_{2^{\cdot}}^{-}$), and peroxynitrite releases were also determined. Lipopolysaccharide increased EFS-induced vasoconstriction at 2 h. This increase was lower after 5-h preincubation. N^{ω}-nitro-L-arginine methyl ester increased vasoconstrictor response only in control segments. Vasodilator response to DEA-NO was increased by LPS after 5-h preincubation and was decreased by $O_{2^{\cdot}}^{-}$ scavenger tempol. Basal NO release was increased by LPS. Electrical field stimulation–induced NO release was reduced by LPS compared with control conditions. Lipopolysaccharide exposure increased both $O_{2^{\cdot}}^{-}$ and peroxynitrite release. Vasoconstriction to exogenous NA was markedly increased by LPS compared with control conditions after 2-h incubation and remained unchanged after 5-h incubation. Short-term exposure of rat mesenteric arteries to LPS produced a time-dependent enhanced contractile response to EFS. The early phase (2 h) was associated to a reduction in NO from neuronal NO synthase and an enhanced response to NA. After 5 h of LPS exposure, this enhancement was reduced, because of restoration of the adrenergic component and maintenance of the nitrergic reduction.

KEYWORDS—LPS, rat mesenteric artery, adrenergic innervation, nitrergic innervation

INTRODUCTION

Endotoxic shock is characterized by hypotension, vascular collapse, and multiple organ failure (1). Several mechanisms have been proposed to account for this syndrome at the vascular level: activation of ATP-sensitive potassium channels in the plasma membrane of vascular smooth muscle, activation of the inducible form of nitric oxide (NO) synthase (NOS), vasopressin deficiency, altered catecholamine release, and vasomotor response (2–6).

Nitric oxide is a main participant in the hypotension associated with vascular collapse in endotoxic shock. Nitric oxide can be produced from the three major NOS isoforms: neural NOS (nNOS), endothelial NOS, and inducible NOS (iNOS) (7, 8). Thus, inhibition of NOS to prevent excessive NO release presents an experimental therapeutic approach to counteract hypotension in shock (9, 10). It is generally accepted that NO from iNOS and endothelial NOS plays a main detrimental role in sepsis-induced multiple organ dysfunction (11–14). Studies investigating effects of lipopolysaccharide (LPS) on nNOS have been performed in different tissues, mainly from central nervous system, yielding a variety of results (15–17). However, studies on the participation in the effects of LPS of NO from nNOS in the regulation of vasomotor tone are scarce and report different results (18, 19).

Arterial tone is regulated by the central nervous system, perivascular innervation, myogenic mechanisms, and endothelial and humoral factors, including cytokines. Perivascular innervation plays a principal role in the regulation of vascular tone, specifically in certain vessels such as the mesenteric vascular bed (20). Neural control of mesenteric artery is mediated by adrenergic and nitrergic components (21–23), which are affected by several pathophysiological situations (24). It has been previously shown that exposure of rat mesenteric segments to LPS results in induction of iNOS associated with increased NO formation and noradrenaline (NA) hyporeactivity (25). Induction and activation of nNOS but not iNOS by LPS have been described in oligodendrocytes (26). This activation resulted in peroxynitrite accumulation and protein tyrosine nitration in oligodendrocytes, resulting in reduced cell viability. Most of the studies investigating the role of NO in vascular dysfunction under LPS exposure are focused on a chronic or late phase (27, 28), whereas the results obtained in acute or short-term studies are inconclusive because of the different experimental conditions (29).

Because there is no functional analysis determining integrated effects of adrenergic and nitrergic alterations under LPS exposure, the aim of the present study was to investigate the impact of LPS on the neuronal control of mesenteric vascular tone as well as the mechanisms involved.

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No conflict of interests exists.

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MATERIALS AND METHODS

Animals

Male Wistar rats (3 months old) were obtained from the Animal Quarters of the Universidad Autónoma de Madrid and housed in the Animal Facility of the Universidad Autónoma de Madrid (registration no. EX-021U) in accordance with guidelines 609/86 of the E.E.C., R.D. 233/88 of the Ministerio de Agricultura, Pesca y Alimentación of Spain, and Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication 85.23, revised 1985). All experimental procedures involving animal use were approved by the Ethics Committee of the Universidad Autónoma de Madrid. Rats were housed at a constant room temperature, humidity, and light cycle (12:12-hour light-dark cycle) with free access to tap water and fed with standard rat chow *ad libitum*.

Animals were killed by CO_2 inhalation; the first branch of the mesenteric artery was carefully dissected, cleaned of connective tissue, and placed in Krebs-Henseleit solution (KHS, in mmol/L: NaCl 115, $CaCl_2$ 2.5, KCl 4.6, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 25, glucose 11.1, Na_2EDTA 0.03) at 4°C. Effects of LPS (10 μg/mL) incubation were studied at two time points: 2 h (phase 1) or 5 h (phase 2). The dose of LPS was selected from pilot studies in which 1-, 10-, and 20-μg/mL doses were assayed. The intermediate dose was chosen because it produced the most reliable response in the alteration of vasomotor response to electrical field stimulation (EFS).

Vascular reactivity

The method used for isometric tension recording has been described in full elsewhere (30). Two parallel stainless steel pins were introduced through the lumen of the vascular segment: one was fixed to the bath wall, and the other connected to a force transducer (Grass FTO3C; Quincy, Mass); this, in turn, was connected to a model 7D Grass polygraph. For EFS experiments, segments were mounted between two platinum electrodes 0.5 cm apart and connected to a stimulator (Grass, model S44) modified to supply adequate current strength. Segments were suspended in an organ bath containing 5 mL of KHS at 37°C and continuously bubbled with a 95% O_2 to 5% CO_2 mixture (pH 7.4). Experiments were performed in endothelium-denuded segments to eliminate the main source of vasoactive substances, including endothelial NO. This avoided possible actions of different drugs on endothelial cells that could lead to misinterpretation of results. Endothelium was removed by gently rubbing the luminal surface of the segments with a thin wooden stick. The segments were subjected to a tension of 0.5 g, which was readjusted every 15 min during a 90-min equilibration period before drug administration. After this, the vessels were exposed to 75 mmol/L KCl, to check their functional integrity. Endothelium removal did not alter the contractions elicited by 75 mmol/L KCl. After a washout period, the absence of vascular endothelium was tested by the inability of 10 μmol/L acetylcholine to relax segments precontracted with NA.

Frequency-response curves to EFS (1, 2, 4, and 8 Hz) were performed. The parameters used for EFS were 200 mA, 0.3 ms, 1 to 8 Hz, for 30 s with an interval of 1 min between each stimulus, the time required to recover basal tone. A washout period of at least 1 h was necessary to avoid desensitization between consecutive curves. Three successive frequency-response curves separated by 1-h intervals produced similar contractile responses.

To analyze the participation of NO in the EFS-induced response in segments from control and LPS-incubated mesenteric segments, 0.1 mmol/L N^{ω}-nitro-L-arginine methyl ester (L-NAME), a nonspecific inhibitor of NOS, was added to the bath 30 min before performing the second frequency-response curve.

The vasodilator response to the NO donor diethylamine NONOate (DEA-NO, 0.1 nmol/L to 0.1 mmol/L) and to peroxynitrite (0.1 μmol/L to 1 mmol/L) and the vasoconstrictor response of exogenous NA (1 nmol/L to 10 μmol/L) were also determined in control and LPS-incubated arteries. To assess the participation of superoxide anion in DEA-NO–induced vasodilation, 0.1 mmol/L tempol, a superoxide anion scavenger, was added to the bath before the frequency-response curve to DEA-NO was performed.

Noradrenaline release

Endothelium-denuded segments of rat mesenteric arteries from all experimental groups were preincubated for 30 min in 5 mL of KHS at 37°C and continuously gassed with a 95% O_2 to 5% CO_2 mixture (stabilization period). This was followed by two washout periods of 10 min in a 0.4 mL KHS bath. Then, the medium was collected to measure basal release. Next, the organ bath was refilled, and cumulative EFS periods of 30 s at 1, 2, 4, and 8 Hz were applied at 1-min intervals. Afterward, the medium was collected to measure EFS-induced neurotransmitter release.

Noradrenaline release was measured using Noradrenaline Research EIA (Labor Diagnostica Nord, GmbH and Co, KG, Nordhon, Germany). Assays were performed following the manufacturer's instructions. Results were expressed as ng NA/mL mg tissue.

NO release

Nitric oxide release was measured using fluorescence emitted by the fluorescent probe 4,5-diaminofluorescein (DAF-2), as previously described (31). Endothelium-denuded mesenteric arteries from all experimental groups were subjected to a 60-min equilibration period in HEPES buffer (in mmol/L: NaCl, 119; HEPES, 20; $CaCl_2$, 1.2; KCl, 4.6; $MgSO_4$, 1; KH_2PO_4, 0.4; $NaHCO_3$, 5; glucose, 5.5; Na_2HPO_4, 0.15; pH 7.4) at 37°C. Arteries were incubated with 2 μmol/L DAF-2 for 30 min. The medium was then collected to measure basal NO release. Once the organ bath was refilled, cumulative EFS periods of 30 s at 1, 2, 4, and 8 Hz were applied at 1-min intervals. Afterward, the medium was collected to measure EFS-induced NO release. The fluorescence of the medium was measured at room temperature using a spectrofluorometer (LS50, FL WINLAB Software; Perkin Elmer Instruments, Whaltmann, Mass) with excitation wavelength set at 492 nm and emission wavelength at 515 nm.

The EFS-induced NO release was calculated by subtracting basal NO release from that evoked by EFS. Also, blank sample measures were collected in the same way from segment-free medium to subtract background emission. Some assays were performed in the presence of 0.1 mmol/L L-NAME; 0.1 mmol/L 7NI, the specific nNOS inhibitor; 1 μmol/L 1400 W, the specific iNOS inhibitor; or 0.1 μmol/L tetrodotoxin (TTX), a nerve impulse blocker. The amount of NO released was expressed as arbitrary units/mg tissue.

Detection of superoxide anions

Superoxide anion levels were measured using lucigenin chemiluminescence, as previously described (32). Endothelium-denuded mesenteric segments from all experimental groups were rinsed in KHS for 30 min, equilibrated for 30 min in HEPES buffer at 37°C, transferred to test tubes that contained 1 mL HEPES buffer (pH 7.4) containing lucigenin (5 μmol/L), and then kept at 37°C. The luminometer was set to report arbitrary units of emitted light; repeated measurements were collected for 5 min at 10-s intervals and averaged. 4,5-Dihydroxy-1,3-benzene-disulfonic acid "Tiron" (10 mmol/L), a cell-permeant, nonenzymatic superoxide anion scavenger, was added to quench the superoxide anion–dependent chemiluminescence. Calculations were performed subtracting the lucigenin chemiluminescence obtained in the presence of Tiron from that obtained in its absence. Blank measures were collected in the same way without mesenteric segments to subtract background emission.

Peroxynitrite detection

Peroxynitrite levels were measured using fluorescence emitted by the fluorescent probe dihydrorhodamine 123. Endothelium-denuded mesenteric arteries from all experimental groups were subjected to a 30-min equilibration period in phosphate-buffered saline solution (in mmol/L: NaCl, 137; KCl, 2.7; $Na_2HPO_4 \cdot 2H_2O$, 10; KH_2PO_4, 2; pH 7.4) at 37°C. Arteries were incubated with 5 μmol/L dihydrorhodamine 123 for 30 min. The medium was then collected to measure basal peroxynitrite release. Once the organ bath was refilled, cumulative EFS periods of 30 s at 1, 2, 4, and 8 Hz were applied at 1-min intervals. Afterward, the medium was collected to measure EFS-induced peroxynitrite release. The fluorescence of the medium was measured at room temperature using a spectrofluorometer (LS50, FL WINLAB Software; Perkin Elmer Instruments) with excitation wavelength set at 500 nm and emission wavelength at 536 nm. Some assays were performed in the presence of 0.1 mmol/L tempol or 0.1 μmol/L TTX. The amount of peroxynitrite released was expressed as arbitrary units/mg tissue. Blank sample measures were collected in the same way from segment-free medium to subtract background emission. The amount of peroxynitrite released was expressed as arbitrary units/mg tissue.

nNOS and phosphorylated nNOS expression

Western blot analysis of nNOS and phosphorylated nNOS (P-nNOS) expression was performed as previously described (32). For these experiments, we used mouse monoclonal nNOS antibody (1:1,000; BD Transduction Laboratories, Madrid, Spain) and rabbit polyclonal P-nNOS antibody (1:2,000; Abcam, Cambridge, UK).

Drugs used

Lipopolysaccharide serotype 055:B5, L-NA hydrochloride, acetylcholine chloride, diethylamine NONOate diethylammonium salt, TTX, tempol, L-NAME hydrochloride, 1400 W, phentolamine, lucigenin, Tiron, and DAF-2 (Sigma-Aldrich, Madrid, Spain) were used. Stock solutions (10 mmol/L) of drugs were made in distilled water, except for NA, which was dissolved in a NaCl (0.9%)–ascorbic acid (0.01% wt/vol) solution. These solutions were kept at −20°C, and appropriate dilutions were made in KHS on the day of the experiment.

Data analysis

The responses elicited by EFS and NA were expressed as a percentage of the initial contraction elicited by 75 mmol/L KCl for comparison between control and LPS-treated arteries. The relaxation induced by DEA-NO was expressed as a percentage of the initial contraction elicited by NA (control: 1,095.546 ± 10.5 mg; LPS 2 h: 1,104.97 ± 12.7 mg; LPS 5 h: 1,074.23 ± 19.54 mg; $P > 0.05$). Results are given as mean ± SEM. Statistical analysis was done by comparing the curve obtained in the presence of the different substances with the previous or control curve by means of repeated-measure analysis of variance followed by Bonferroni post hoc test, using the GraphPad Prism 5.0 software (GraphPad Software, Inc, San Diego, Calif). For NO, superoxide anion, peroxynitrite, and NA release data, the statistical analysis was done using one-way analysis of variance followed by Newman-Keuls post hoc test for unpaired experiments. $P < 0.05$ was considered significant.

RESULTS

Response to KCl

Response of mesenteric segments to KCl (75 mmol/L) was similar in control and segments exposed to LPS (2 and 5 h) (control: 1,909 ± 183 mg; LPS 2 h: 2,105 ± 213 mg; LPS 5 h: 1,879 ± 189 mg).

Effect of LPS exposure in vasomotor response to EFS

As previously shown (16), EFS induced a frequency-dependent contraction that was maintained without modifications over the entire study period (5 h) (Fig. 1A). Two hours of exposure to LPS induced an increased ($P < 0.05$) contractile response, which was observed at all frequencies (1–8 Hz). In contrast, after 5 h of LPS incubation, a reduction ($P < 0.05$; compared with the 2-h period) in the EFS-induced contraction was observed at all frequencies (Fig. 1B).

Incubation of mesenteric arteries with the unspecific NOS inhibitor L-NAME (0.1 mmol/L) increased ($P < 0.05$) the contractile response at all frequencies only in control segments (Fig. 2A). Exposure to LPS in the presence of L-NAME did not alter vasoconstrictor response at any frequency (1–8 Hz) or exposure period (2 and 5 h) (Fig. 2, B and C).

NO relaxation and release

Relaxation of mesenteric segments to exogenous DEA-NO was not altered in the presence of LPS compared with control conditions after 2 h of LPS incubation, whereas relaxation was increased by LPS after 5-h incubation (Fig. 3A). Preincubation with 0.1 mmol/L tempol did not modify DEA-NO vasodilation in control or 2 h-incubated LPS segments, (Fig. 3, B and C), although it decreased after 5-h incubation with LPS (Fig. 3D).

Basal release of NO increased ($P < 0.05$) in both phases (Fig. 4A). In contrast, during EFS stimulation, the increase in NO release was markedly reduced ($P < 0.05$) compared with control conditions at both time periods (Fig. 4B).

Basal NO release was markedly reduced in the presence of the iNOS inhibitor 1400 W over the studied period, but not in the presence of the nNOS specific inhibitor, 7NI (Fig. 4A). The EFS-induced NO release was almost abolished in presence of 7NI (Fig. 4B), the unspecific NOS inhibitor, L-NAME (data not shown), or the nerve impulse blocker TTX (Fig. 4B). In contrast, release was not modified after exposure to 1400 W (Fig. 4B).

nNOS expression

Neural NOS protein expression was not modified by LPS exposure at any phase. However, P-nNOS expression decreased after 2 and 5 h of LPS exposure (Fig. 4C).

Oxidative and nitrosative stress

Lipopolysaccharide exposure produced a progressive increase in superoxide anions, which reached significance after 5 h of exposure (Fig. 5A). Similarly, although basal peroxynitrite levels were undetectable, EFS-induced peroxynitrite levels also increased over the studied period (Fig. 5B) and were almost abolished in presence of superoxide anion scavenger tempol (Fig. 5B) or the nerve impulse blocker TTX (Fig. 5B) in segments from all experimental groups.

Exogenous peroxynitrite induced a concentration-dependent response that was not altered by LPS at any time of exposure (Fig. 5C).

NA contraction and release

Contraction of mesenteric segments to exogenous NA in the presence of LPS was markedly increased ($P < 0.05$) compared with control conditions at 2 h and remained unchanged after 5 h of LPS exposure (Fig. 6A).

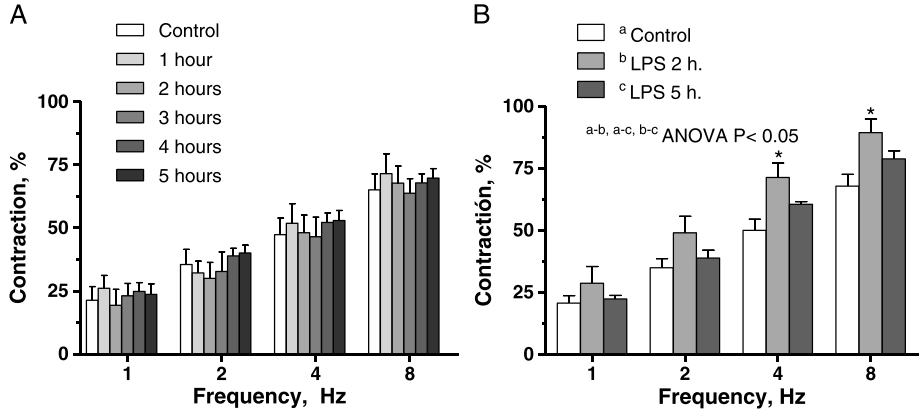

Fig. 1. **Effect of LPS on EFS-induced vasoconstrictor**. A, Time-dependent EFS-induced contractile response in mesenteric segments from Wistar rats. B, Vasoconstrictor response to EFS in control and LPS-incubated (10 μg/mL, 2 and 5 h) mesenteric arteries. Results (mean ± SEM) were expressed as a percentage of the initial contraction elicited by KCl. *$P < 0.05$ vs. control segments for each frequency (Bonferroni test). n = 10 animals each group.

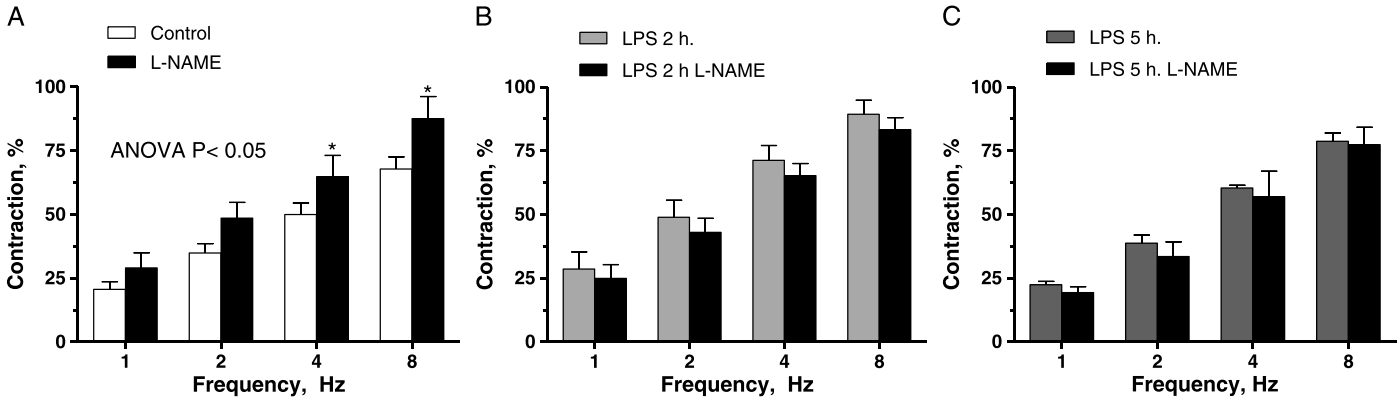

FIG. 2. **Influence of neuronal NO on EFS-induced vasoconstriction**. Effect of preincubation with 0.1 mmol/L L-NAME on the vasoconstrictor response to EFS in control (A) and LPS-incubated (10 μg/mL) segments for 2 h (B) and 5 h (C). Results (mean ± SEM) were expressed as a percentage of the initial contraction elicited by KCl. *$P < 0.05$ vs. control situation for each frequency (Bonferroni test). n = 10 animals each group.

Lipopolysaccharide exposure did not modify NA release during EFS stimulation compared with control conditions at any exposure time (Fig. 6B).

DISCUSSION

The study shows that short-term exposure of rat mesenteric arteries to LPS induced an increased contractile response to EFS that exhibited a time-dependency pattern. In the early phase (2 h), an enhanced constrictor response was dependent on a reduction in NO from nNOS and an enhanced response to NA. After 5 h of LPS exposure, this enhancement was reduced because of the restoration of the adrenergic component, although the nitrergic reduction continued.

Perivascular innervation plays a pivotal role in the regulation of mesenteric blood flow (20, 23). Adrenergic and nitrergic innervations, among other factors, have been reported in rat mesenteric artery, where they seem to play a principal role (21, 23). The interplay between both types of innervation that has been described in the regulation of blood flow under different pathological circumstances could have multiple hemodynamic consequences (33, 34). Endotoxic shock is characterized by hypotension, vascular collapse, and multiple organ failure including gastrointestinal tract (1, 7). Previous studies under a variety of experimental conditions have already shown that NO plays a pivotal role in the consequences of endotoxic shock (9, 27). Under these conditions, increased NO production is mainly due to enhanced activity and expression of iNOS in a variety of tissues, including the vascular wall (7, 25, 27).

In addition, alterations of NO produced in endotoxic shock by nNOS in several tissues have also been studied, yielding a variety of results. Reduced nNOS expression produced by endotoxins in the neurons from the myenteric plexus of rabbit duodenum has been reported (17), whereas increased nNOS protein levels in skeletal muscle during sepsis have also been reported (16). Increases in nNOS and iNOS gene expression, with different time courses, were observed in the paraventricular nucleus 2 h after LPS injection in rats (15). Systemic administration of LPS in rats induced a transcriptional upregulation of nNOS in the brainstem dorsal vagal complex (35). On the contrary, downregulation of nNOS in cardiomyocytes from rats treated with LPS has also been reported (36).

Effect of 2 h exposure to LPS

Nitric oxide synthesis could be due to the activation of different NOS isoforms in the mesenteric artery wall. In our study, EFS-induced NO release was almost abolished in the presence of TTX, confirming its neuronal origin. In the presence of LPS, basal NO release increased, although EFS-induced NO release was reduced. Although basal release of NO was not altered by the specific nNOS inhibitor 7NI, it was reduced by the inhibitor of iNOS 1400 W, indicating its inducible origin. This result confirms the important role of NO from iNOS in the vasodilation induced by LPS. In turn, EFS-stimulated NO

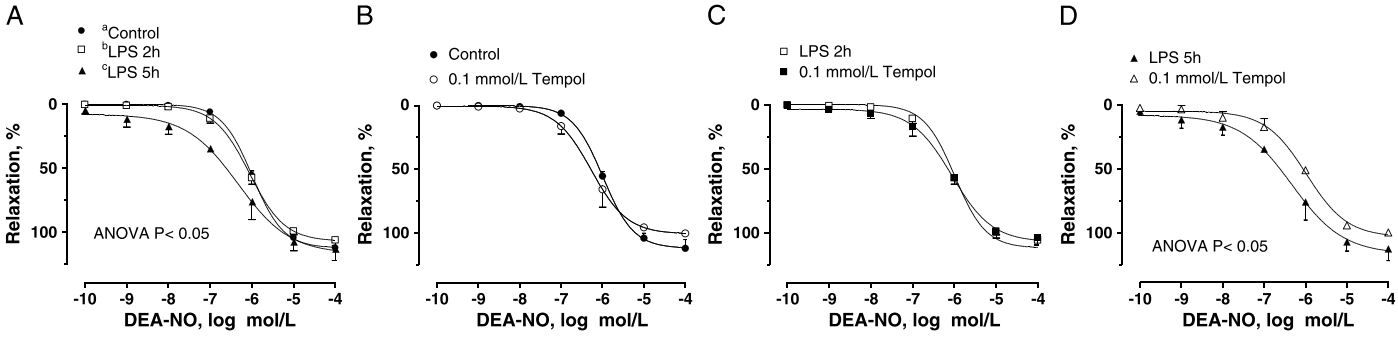

FIG. 3. **Effect of LPS on DEA-NO induced vasodilation**. A, Vasodilator response to DEA-NO in control and LPS-incubated (10 μg/mL, 2 and 5 h) mesenteric arteries. Effect of preincubation with 0.1 mmol/L tempol on the vasodilator response elicited by DEA-NO in segments from control (B) and LPS-incubated (10 μg/mL) segments at 2 h (C) and 5 h (D). Results (mean ± SEM) are expressed as a percentage of the initial contraction elicited by exogenous NA. *$P < 0.05$ vs. control conditions for each dose (Bonferroni test). n= 8 animals each group.

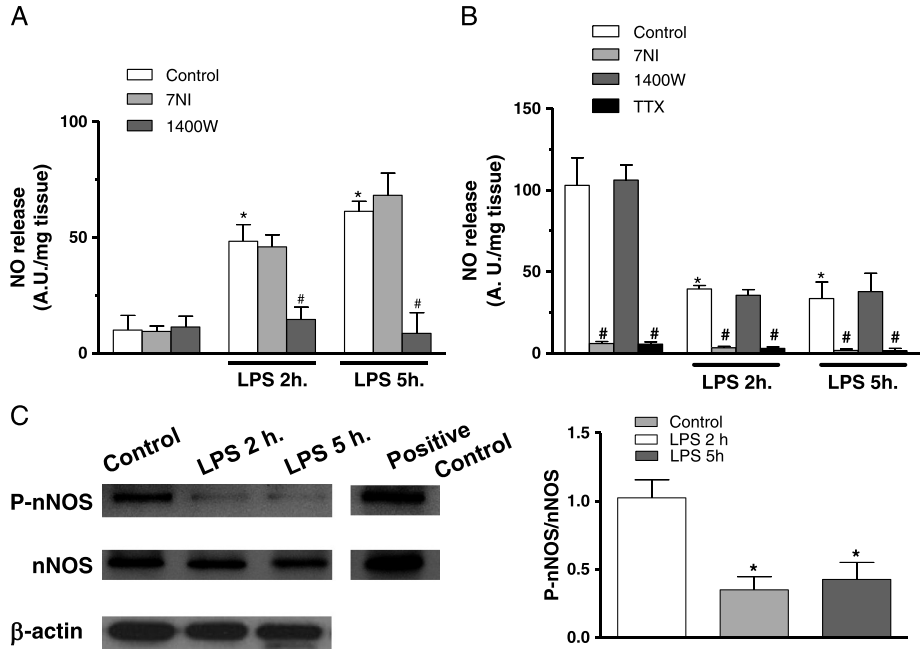

Fig. 4. **Effect of LPS on NO release and nNOS expression.** Effect of preincubation with 0.1 mmol/L 7NI, 1 μmol/L 1400 W, or 0.1 μmol/L TTX on basal (A) NO release and EFS-induced (B) NO release in control and LPS-incubated mesenteric segments for 2 and 5 h. Results (mean ± SEM) are expressed as arbitrary units (AU)/ mg tissue. $^{*}P < 0.05$ vs. control segments; $^{\#}P < 0.05$ vs. release without specific inhibitor. n = 8 animals per group. C, Effect of LPS incubation (10 μg/mL, 2 or 5 h) on nNOS and P-nNOS expression. The blot is representative of six separate segments from each group. Results (mean ± SEM) are expressed as the ratio of the signal obtained for P-nNOS and the one obtained for nNOS. $^{*}P < 0.05$ vs. control.

release in the absence and presence of LPS was abolished by 7NI but was not modified by 1400 W. This indicates that nNOS is responsible for the NO release induced by EFS and rules out the participation of iNOS, thus confirming previous findings (37).

To explore possible mechanisms involved in the reduced NO release, nNOS expression and activation to its phosphorylated form were studied. Neural NOS protein expression remained unaltered, whereas P-nNOS was markedly diminished over the study period. Thus, it could be proposed that a reduction of nNOS activity seems to cause the aforementioned reduced NO release.

Incubation of mesenteric arteries with the unspecific NOS inhibitor L-NAME in control conditions produced an increased EFS-vasoconstrictor response, but this was not observed during 2-h LPS exposure. In addition, the participation of other components different than nitrergic innervation in the increased contractile response to EFS cannot be ruled out.

Previous studies have already shown differential participation of the adrenergic system in the presence of LPS or in endotoxic shock. Mesenteric arteries from pigs showed an elevated contractile response to NA 3 h after induction of endotoxic shock with LPS, a response that is probably associated to changes in endothelial factors (38). Enhanced NA release from sympathetic nerves has been reported, and it may play a role in the preservation of neurogenic response after LPS treatment despite evidence of the induction of NOS (5). Other authors have shown hyporeactivity to NA or methoxamine in mesenteric arteries exposed to LPS or in arteries from rats infused with LPS (25, 39). This latter effect was not due to a generalized inability of the smooth muscle to contract, as demonstrated by the unaltered response to KCl, and does not appear to involve abnormalities in calcium mobilization or entry (39). On the contrary, alterations in calcium handling and a decreased sensitivity of myofilaments to calcium have been proposed as another possible mechanism contributing to the effects of LPS in mesenteric arteries (40). In our study, dose-response contraction of mesenteric segments to exogenous NA in the presence of LPS was markedly increased at 2 h of LPS exposure. Thus, the observed increase in NA response could be due to postjunctional modifications (ion channels, intracellular mediators, etc.). In addition, a facilitating prejunctional mechanism has been reported that may preserve sympathetic function in endotoxemia (5). Thus, the observed increased contraction to NA does not rule out the possibility of a modified NA release. The results show that LPS exposure did not modify NA release at the basal point or during EFS stimulation compared with control conditions. Thus, the observed increased contraction to EFS at 2 h seems to be due to an enhanced NA response together with a reduction in NO.

Effect of 5-h exposure to LPS

The study also shows that 5-h exposure of rat mesenteric arteries to LPS induced an increased contractile response to EFS, a response that was lower than that observed at 2 h, as previously reported (41). Basal and stimulated NO release at 5 h was similar to that found at 2 h. Incubation with 7NI and 1400 W also showed a similar pattern to the one observed under both basal and stimulated conditions at 2 h. Similarly to findings at 2 h, nNOS protein expression remained unaltered, whereas P-nNOS was markedly diminished over the

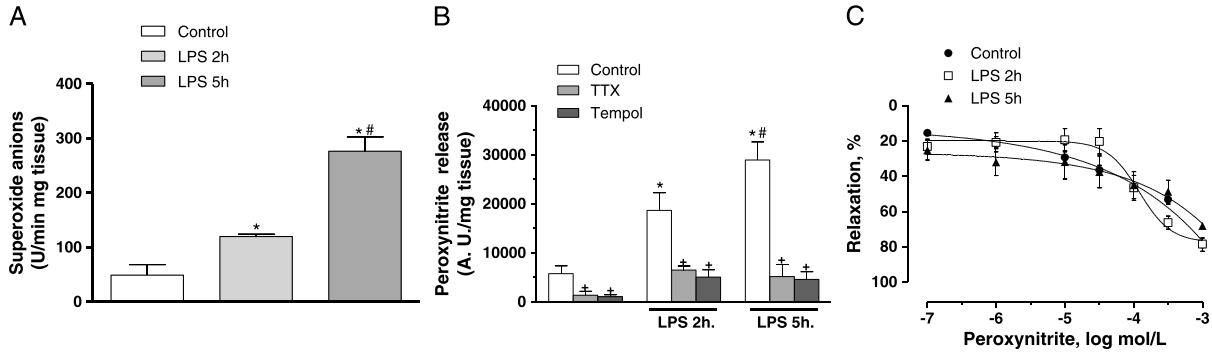

FIG. 5. **Effect of LPS on oxidative and nitrosative stress**. A, Superoxide anion release from control and LPS-incubated segments (10 μg/mL, 2 or 5 h). Results (mean ± SEM) are expressed as chemiluminescence units (U)/min mg tissue. $^{*}P < 0.05$ vs. control. n = 5 animals per group. B, Effect of preincubation with 0.1 μmol/L TTX or 0.1 mmol/L tempol on peroxynitrite formation in control and LPS-incubated mesenteric segments (10 μg/mL, 2 or 5 h). Results (mean ± SEM) are expressed as arbitrary units (AU)/ mg tissue. $^{*}P < 0.05$ vs. control segments; $^{\#}P < 0.05$ vs. 2 h-LPS incubated segments; $^{+}P < 0.05$ vs. release without specific inhibitor. n = 6 animals per group. C, Vasodilator response to exogenous peroxynitrite in control and LPS-incubated (10 μg/mL, 2 and 5 h) mesenteric arteries. Results (mean ± SEM) are expressed as a percentage of the initial contraction elicited by exogenous NA. n = 5 animals each group.

longer study period. Furthermore, L-NAME incubation did not induce any modification, as had been observed at 2 h. Collectively, these results indicate that nitrergic innervation remained unaltered over the studied period, and consequently the observed reduction compared with the previous early phase should be due to the adrenergic component. Our results show a normalization of NA response to levels similar to those observed under control conditions and reflect the lower increment observed at 5 h. However, we do not have any feasible explanation for this effect.

Oxidative and nitrosative stress

Previous studies showed that endotoxemia increased superoxide anion formation, possibly contributing to NO degradation (42, 43). Our results showed a progressive (2 and 5 h) increase in superoxide anions in mesenteric arteries exposed to LPS. This effect was accompanied by a parallel increase in peroxynitrites, indicating a time-dependent reduction in NO bioavailability over the course of the study. In addition, when either neuronal NO release or superoxide anion was eliminated by the respective presence of either TTX or tempol, the rhodamine fluorescent signal dropped. This confirmed that the signal observed in the absence of these drugs is due to peroxynitrite formation originated from neuronal NO.

To determine whether superoxide anions could be an additional mechanism that reduced NO availability in the presence of LPS, we analyzed the vasodilator response of mesenteric segments to the NO donor DEA-NO. The results demonstrated no differences in the response to the NO donor in the presence or absence of LPS after 2 h. Similarly, Cuzzocrea et al. (44) showed no difference in vasorelaxation produced by sodium nitroprusside in aortic rings from LPS-treated rats. However, LPS increased DEA-NO–induced vasodilation after a 5-h preincubation, and the effect was reversed by tempol. The present results, like previous studies from our group, show a vasodilatory effect of peroxynitrite in this vascular bed (45). Consequently, the increase in peroxynitrite levels may contribute to the increase in relaxation induced by DEA-NO.

It is well known that LPS stimulates cytokine production and release in several tissues and that cytokines are able to alter adrenergic and nitrergic function similarly to the manner observed in our results (46). Thus, the effects of LPS observed in our experimental conditions might also be mediated by cytokines. Identification of cytokine participation could be the subject of future studies.

In summary, short-term exposure of rat mesenteric arteries to LPS produced a time-dependent enhanced contractile response to EFS. The early phase (2 h) was associated to a reduction in NO from nNOS and an enhanced response to NA. After 5 h of LPS exposure, this enhancement was reduced, because of restoration of the adrenergic component and maintenance of the nitrergic reduction.

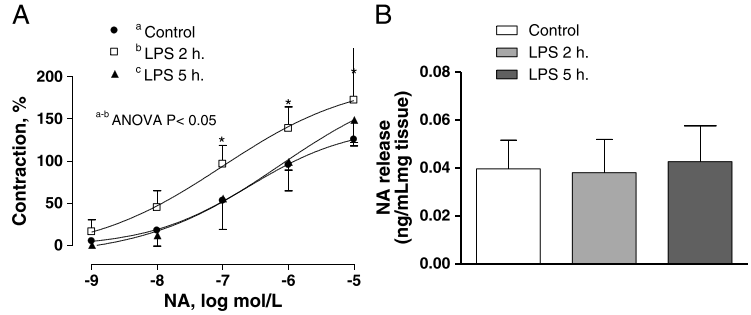

FIG. 6. **Effect of LPS on NA-induced vasoconstriction and release**. A, Vasoconstrictor response to exogenous NA in control and LPS-incubated (10 μg/mL, 2 or 5 h) mesenteric arteries. Results (mean ± SEM) are expressed as a percentage of the previous contraction elicited by KCl. n = 5 animals each group. $^{*}P < 0.05$ vs. control groups (Bonferroni test). B, Electrical field stimulation–induced NA release in mesenteric segments from control and LPS-incubated mesenteric segments. Results (mean ± SEM) are expressed as ng NA/mL mg tissue. n = 5 animals per group.

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Artículo 8: Opposite effect of mast cell stabilizers ketotifen and tranilast on the vasoconstrictor response to electrical field stimulation in rat mesenteric artery.

Sastre E, Caracuel L, Xavier FE, Balfagón G, Blanco-Rivero J**.**

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Opposite Effect of Mast Cell Stabilizers Ketotifen and Tranilast on the Vasoconstrictor Response to Electrical Field Stimulation in Rat Mesenteric Artery

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Abstract

Objectives: We analyzed whether mast cell stabilization by either ketotifen or tranilast could alter either sympathetic or nitrergic innervation function in rat mesenteric arteries.

Methods: Electrical field stimulation (EFS)-induced contraction was analyzed in mesenteric segments from 6-month-old Wistar rats in three experimental groups: control, 3-hour ketotifen incubated (0.1 αmol/L), and 3-hour tranilast incubated (0.1 mmol/L). To assess the possible participation of nitrergic or sympathetic innervation, EFS contraction was analyzed in the presence of non-selective nitric oxide synthase (NOS) inhibitor L-NAME (0.1 mmol/L), α-adrenergic receptor antagonist phentolamine (0.1 μmol/L), or the neurotoxin 6-hydroxydopamine (6-OHDA, 1.46 mmol/L). Nitric oxide (NO) and superoxide anion (O_2^{-}) levels were measured, as were vasomotor responses to noradrenaline (NA) and to NO donor DEA-NO, in the presence and absence of 0.1 mmol/L tempol. Phosphorylated neuronal NOS (P-nNOS) expression was also analyzed.

Results: EFS-induced contraction was increased by ketotifen and decreased by tranilast. L-NAME increased the vasoconstrictor response to EFS only in control segments. The vasodilator response to DEA-NO was higher in ketotifen- and tranilast-incubated segments, while tempol increased vasodilator response to DEA-NO only in control segments. Both NO and O_2^{-} release, and P-nNOS expression were diminished by ketotifen and by tranilast treatment. The decrease in EFS-induced contraction produced by phentolamine was lower in tranilast-incubated segments. NA vasomotor response was decreased only by tranilast. The remnant vasoconstriction observed in control and ketotifen-incubated segments was abolished by 6-OHDA.

Conclusion: While both ketotifen and tranilast diminish nitrergic innervation function, only tranilast diminishes sympathetic innnervation function, thus they alter the vasoconstrictor response to EFS in opposing manners.

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Introduction

Arterial tone is regulated by the central nervous system, perivascular innervation and myogenic mechanisms, as well as by endothelial and humoral factors. Perivascular innervation plays a principal role in the regulation of vascular tone, specifically in certain vessels such as the mesenteric vascular bed [1,2], where blood flow is approximately 20-30% of the total cardiac output [3]. Inadequate mesenteric blood flow and tissue perfusion can produce relevant haemodynamic changes [3–5]. This regulation involves sympathetic and nitrergic innervations (1,2), which mainly release mainly noradrenaline (NA) or ATP from sympathetic nerve terminals [6], and nitric oxide (NO) from nitrergic innervation [7,8].

An interaction between mast cells and nerve endings has been observed in the gastrointestinal tract [9] and cardiac tissue [10]. When stimulated, these cells degranulate, subsequently releasing a variety of mediators, such as neutral proteases, growth factors, cytokines and chemokines, as well as vasoactive substances like serotonin, histamine, leukotrienes and prostaglandins [11,12], which can affect perivascular mesenteric innervation function.

Ketotifen and tranilast are effective mast cell stabilizer agents widely used in the management of allergic and inflammatory disorders. Both drugs block a calcium channel essential for mast cell degranulation, thereby stabilizing the cell membrane[12–15]. . Additionally, ketotifen and tranilast can also induce different additional effects apart from mast cell stabilization, effects that could induce different alterations in perivascular nerve function [16–18].

Based on these considerations, the aim of this study was to analyze the possible different effects of ketotifen and tranilast on sympathetic and nitrergic function in rat mesenteric arteries, as well as the mechanism/s implicated.

Methods

Ethics Statement

All animals were housed in the Animal Facility of the Universidad Autónoma de Madrid (Registration number EX-021U) in accordance with directives 609/86 of the E.E.C., R.D. 233/88 of the Ministerio de Agricultura, Pesca y Alimentación of Spain, and Guide for the Care and Use of Laboratory Animals published by the USA National Institutes of Health (NIH publication No. 85.23, revised 1985). The experimental protocol was approved by the Ethics Committee of the Universidad Autónoma de Madrid.

Animals

We used 6 month-old male Wistar rats. Rats were sacrificed by CO_2 inhalation followed by decapitation; the first branch of the mesenteric artery was carefully dissected out, cleaned of connective tissue and placed in Krebs–Henseleit solution (KHS, in mmol/L : NaCl 115, $CaCl_2$ 2.5, KCl 4.6, KH_2PO_4 1.2, $MgSO_4$. $7H_2O$ 1.2, $NaHCO_3$ 25, glucose 11.1, Na $_2$EDTA 0.03) at 4°C. Some samples were immediately frozen in liquid nitrogen and stored at -70 ˚C.

Perivascular mast cell detection

Mesenteric arteries were fixed in 4% formaldehyde in phosphate buffered saline solution (PBS, pH=7.4) for 1 hour, cryoprotected with 30% w/v sucrose in PBS (overnight), transferred to a cryomold containing Tissue-Tek OCT embedding medium (20 min) and then immediately frozen in liquid nitrogen. All samples were kept at -70 ˚C until the day of the experiments. Frozen tissue segments were cut into 10 µm thick sections, placed on glass slides and stained with 0.1% Toluidine Blue (3 min) for perivascular mast cell detection. Sections were coverslipped and light microscopy images were taken (Nikon Eclipse TE2000-S (inverted microscope), Nikon DXM1200F (digital camera)).

Vascular Reactivity

The method used for isometric tension recording has been described in full elsewhere [8,19]. Briefly, two parallel stainless steel pins were introduced through the lumen of the vascular segment: one was fixed to the bath wall, and the other connected to a force transducer (Grass FTO3C; Quincy, Mass., USA); in turn, this was connected to a model 7D Grass polygraph. For EFS experiments, segments were mounted between two platinum electrodes 0.5 cm apart and connected to a stimulator (Grass, model S44) modified to supply the appropriate current strength. Segments were suspended in an organ bath containing 5 mL of KHS at 37°C continuously bubbled with a 95% O_2 -5% CO_2 mixture (pH 7.4). Some experiments were performed in endothelium-denuded segments to eliminate the main source of vasoactive substances, including endothelial NO. This avoided possible actions by different drugs on endothelial cells that could lead to misinterpretation of results. Endothelium was removed by gently rubbing the luminal surface of the segments with a thin wooden stick. The segments were subjected to a tension of 0.5 g, which was readjusted every 15 min during a 90-min equilibration period before drug administration. After this, the vessels were exposed to 75 mmol/L KCl to check their functional integrity. Endothelium removal did not alter the contractions elicited by KCl. After a washout period, the presence/absence of vascular endothelium was tested by the ability of 10 µmol/L acetylcholine (ACh) to relax segments precontracted with 1 µmol/L noradrenaline (NA).

Frequency-response curves to EFS (1, 2, 4, 8 and 16 Hz) were performed. The parameters used for EFS were 200 mA, 0.3 ms, 1–16 Hz, for 30 s with an interval of 1 min between each stimulus, the time required to recover basal tone. A washout period of at least 1 h was necessary to avoid desensitization between consecutive curves. Three successive frequency-response curves separated by 1-hour intervals were performed in every segment. EFS responses in the presence of mast cell stabilizers ketotifen (1 µmol/L, 0.1 µmol/L or 10 nmol/L), or tranilast (1 mmol/L, 0.1 mmol/L or 10 µmol/L) were performed to evaluate the possible effect of these drugs on the neural control of vasomotor tone. To analyze a possible time-dependent effect, either ketotifen or tranilast were added to the bath for different incubation periods: 1, 2, and 3 h, before the corresponding frequency-response curves. To evaluate whether the EFS-induced contractile response had a neural origin, the blocker for nerve impulse propagation tetrodotoxin (TTX, 0.1 µmol/L) was added to the bath 30 min in advance.

Vasodilator response to ACh (0.1 nmol/L-10 µmol/L) was tested in endothelium-intact arteries from all experimental groups.

To determine the participation of NO in the EFS-induced response in all experimental groups, 0.1 mmol/L N ^{G}-nitro-L-arginine methyl ester (L-NAME), the unspecific nitric oxide synthase (NOS) inhibitor, or 1 µmol/L 1400W, the specific inducible NOS inhibitor, were added to the bath 30 min before performing the frequency–response curve.

To determine the participation of adrenergic component of sympathetic innervation on the EFS-induced response in control and ketotifen- or tranilast-incubated segments, 1 µmol/L phentolamine, an α-adrenoceptor antagonist, was added to the bath 30 min before performing the frequency-response curve.

The method to deplete sympathetic innervation has been used previously by our group in this artery [20]. Briefly, control, and ketotifen-incubated endothelium-denuded mesenteric segments were incubated at room temperature for 10 minutes in KHS ($NaHCO_3$ and NaH_2PO_4 were omitted, unbuffered

solution) containing 0.02 mmol/L glutathione and 1.46 mmol/L of the neurotoxin 6-hydroxydopamine (6-OHDA). The pH of this solution was adjusted to 4.9 with 0.05 mmol/L NaOH and then the solution was covered with paraffin oil. Subsequently, the arteries were immersed in normal KHS and EFS-induced contraction experiments were performed.

The vasoconstrictor response of exogenous NA (1 nmol/L-10 μmol/L), and the vasodilator response to the NO donor diethylamine NONOate (DEA-NO, 0.1 nmol/L–0.1 mmol/L) were tested in mesenteric segments from all experimental groups. The possible participation of superoxide anions ($O_2^{\cdot-}$) in the vasodilator response to DEA-NO was tested by incubation with 0.1 mmol/L of the $O_2^{\cdot-}$ scavenger tempol.

Histamine and noradrenaline release

To measure histamine and NA release, we used a Histamine Enzyme Immunoassay kit (Spibio, Berlin) and a Noradrenaline Research EIA (Labor Diagnostica Nord, Gmbh & Co., KG), respectively. Endothelium-denuded segments from control, ketotifen-incubated (0.1 μmo/L, 3 hours) or tranilast-incubated (0.1 mmo/L, 3 hours) mesenteric arteries were preincubated in 5 mL of KHS at 37°C and continuously gassed with a 95% O_2-5% CO_2 mixture (stabilization period). This was followed by two washout periods of 10 min in a bath of 0.4 mL of KHS. Then the medium was collected to measure basal histamine or NA release. Next, the organ bath was refilled, and cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz at 1 min intervals were applied. Afterwards, the medium was collected to measure EFS-induced histamine or NA release. The assay was performed following the manufacturer's instructions. Results were expressed as nmol Histamine /mL mg tissue or ng NA/mL mg tissue.

Nitric Oxide Release

Nitric oxide release was determined using the fluorescent probe 4,5-diaminofluorescein (DAF-2), as previously described [21]. Briefly, endothelium-denuded arteries were divided into several experimental groups: control, and segments incubated with ketotifen (0.1 μmol/L, 3 hours), tranilast (0.1 mmol/L, 3 hours), loratadine (1 μmol/L, 30 min) or famotidine (1 μmol/L, 30 min). After an equilibration period of 60 min in HEPES buffer (in mmol/L: NaCl 119; HEPES 20; $CaCl_2$ 1.2; KCl 4.6; $MgSO_4$ 1; KH_2PO_4 0.4; $NaHCO_3$ 5; glucose 5.5; Na_2HPO_4 0.15; pH 7.4) at 37°C, arteries were incubated with 2 μmol/L DAF-2 for 45 min. Then the medium was collected to measure basal NO release. Once the organ bath was refilled, cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz at 1 min intervals were applied. The fluorescence of the medium was measured at room temperature using a spectrofluorimeter (LS50 PerkinElmer Instruments, FL WINLAB Software) with excitation wavelength set at 492 nm and emission wavelength at 515 nm.

The EFS-induced NO release was calculated by subtracting basal NO release from that evoked by EFS. Also, blank samples were collected in the same way from segment-free medium in order to subtract background emission. Some assays were performed in the presence of 0.1 μmol//L TTX, 0.1 mmol/L L-NAME or 0.1 mmol/L 7-NI, a specific nNOS inhibitor, to ensure the specificity of the method. The amount of NO released was expressed as arbitrary units/mg tissue.

nNOs and P-nNOS expression

Western blot analysis of nNOS and phosphorylated nNOS (P–NOS) expression was performed as previously described [22,23]. Rabbit polyclonal antibody against nNOS (1:1000 dilution, Abcam), rabbit polyclonal antibody against P-nNOS (1:1000 dilution, Abcam), and monoclonal anti-β-actin-peroxidase antibody (1:50000, Sigma-Aldrich, Spain) were used. Rat brain homogenates were used as a positive control.

Detection of O_2^-

O_2^- levels were measured using lucigenin chemiluminescence, as previously described [22,24]. Briefly, endothelium-denuded segments of control, ketotifen-incubated (0.1 μmo/L, 3 hours) or tranilast-incubated (0.1 mmo/L, 3 hours) mesenteric arteries were equilibrated for 30 min in HEPES buffer at 37°C, transferred to test tubes that contained 1 mL HEPES buffer (pH 7.4) containing lucigenin (5 μmol/L) and then kept at 37 °C. The luminometer was set to report arbitrary units of emitted light; repeated measurements were collected during 5 min at 10 s intervals and averaged. 4,5-Dihydroxy-1,3-benzene-disulphonic acid ''Tiron'' (10 mmol/L), a cell permeant, non-enzymatic O_2^- scavenger, was added to quench the O_2^--dependent chemiluminescence. Some segments were preincubated with 0.1 mmol/L tempol before the experimental protocol was performed, in order to ensure the specificity of the method. Also, blank measures were collected in the same way without mesenteric segments to subtract background emission.

3-nitrotyrosine (3-NT) detection

3-NT levels were determined using the Nitrotyrosine ELISA kit from Abcam (Cambridge, UK). For this assay, frozen endothelium-denuded segments of control, ketotifen-incubated (0.1 μmo/L, 3 hours) or tranilast-incubated (0.1 mmo/L, 3 hours) mesenteric arteries were homogenized in PBS and centrifuged at 600g for 10 min at 4°C. The supernatant was then collected and used for the assay. 3-NT was measured following the manufacturer's protocol. Results were normalized with protein content, using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Results are expressed as ng 3-NT/mg protein.

Drugs used

L-NA hydrochloride, 6-hydroxydopamine (6-OHDA), ACh chloride, diethylamine NONOate diethylammonium salt, TTX, 1400W, L -NAME hydrochloride, 7- nitroindazole, tempol, phentolamine, DAF-2, lucigenin and tiron were purchased from Sigma-Aldrich (Spain). Stock solutions (10 mmol/L) of drugs were made in distilled water, except for NA, which was dissolved in NaCl (0.9%)-ascorbic acid (0.01% w/v), and 7NI and tempol, which were dissolved in DMSO. These solutions were kept at -20°C and appropriate dilutions were made in KHS on the day of the experiment.

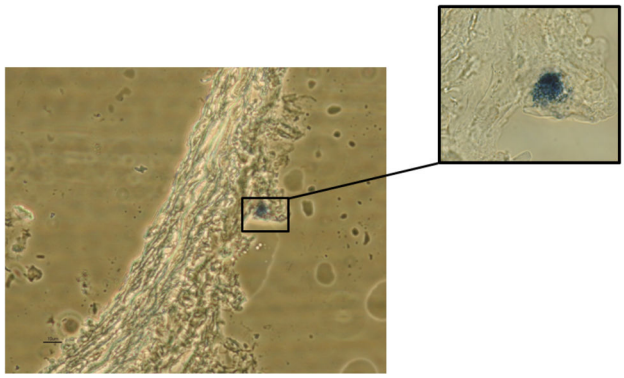

Figure 1

Figure 1. Mast cell localization by toluidine blue staining. Figure is representative of preparations from four rats. Magnification: 400·X and 600 X.

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Data Analysis

The responses elicited by EFS or NA were expressed as a percentage of the initial contraction elicited by 75 mmol/L KCl for comparison between control, ketotifen-incubated and tranilast-incubated segments. The relaxation induced by DEA-NO was expressed as a percentage of the initial contraction elicited by NA (Control: 995.5 ± 14.6 mg; ketotifen: 1002.9 ± 29.41 mg; tranilast: 946.7 ± 28.47 mg, P> 0.05). Results are given as mean ± S.E.M. Statistical analysis was done by comparing the curve obtained in the presence of the different substances with the previous or control curve by means of repeated measure analysis of variance (ANOVA) followed by the Bonferroni post-hoc test. Some results were expressed as differences of area under the curve (dAUC). AUC were calculated from the individual frequency-response plots. For dAUC, histamine, NO and NA release experiments, the statistical analysis was done using one-way ANOVA followed by Newman-Keuls post-hoc test. *P*< 0.05 was considered significant.

Results

Perivascular mast cell detection

Mast cells were detected in the adventitial layer of mesenteric arteries using toluidine blue staining (Figure 1).

Histamine release

Basal histamine release was significantly lower in ketotifen-incubated and tranilast-incubated segments than in control arteries. EFS increased histamine release in all experimental groups, but the increase was greater in control segments. Preincubation with TTX abolished EFS-induced histamine release in all experimental conditions (Table 1).

Table 1. Effect of 0.1 µmol/L ketotifen and 0.1 mmol/L tranilast on basal and EFS-induced histamine and NA release.

	Histamine release		NA release	
	Basal	EFS	Basal	EFS
Control	0.51 ± 0.15	1.19 ± 0.27*	6.65 ± 0.99	9.25 ± 1.13*
Ketotifen	0.15 ± 0.03#	0.38 ± 0.16*#	6.67 ± 0.60	9.33 ± 0.88*
Tranilast	0.19 ± 0.02#	0.52 ± 0.11*#	6.55 ± 0.65	8.89 ± 1.05*
TTX	0.58 ± 0.09	0.66 ± 0.17#	6.37 ± 0.74	6.45 ± 0.87#

This table presents histamine and NA levels released in basal and EFS conditions. Results (means ± SEM) are expressed as nmol histamine /mL mg tissue, or ng NA/mL mg tissue. *P< 0.05 vs basal conditions. #P< 0.05 vs control. n= 6 animals each group.

Vasomotor Response to KCl

In endothelium-intact mesenteric segments, the vasoconstrictor response to 75 mmol/l KCl was similar in all experimental groups (Control, 964.3 ± 59.9 mg, 0.1 µmol/L ketotifen, 913.6 ± 74.5 mg, 0.1 mmol/L tranilast ; 917.8 ± 65.2 mg; P > 0.05; n= 10 each group). Endothelium removal did not alter KCl-induced vasoconstriction (Control, 976.3 ± 67.6 mg, 0.1 µmol/L ketotifen, 912.8 ± 62.7 mg, 0.1 mmol/L tranilast ; 915.5 ± 54.4 mg; P > 0.05; n= 10 each group).

Vascular responses to EFS

The application of EFS induced a frequency-dependent contractile response in endothelium-intact mesenteric segments. Four consecutive EFS curves were performed at 1-h intervals in control mesenteric segments, and induced similar contractions (Figure 2A). Preincubation with 10 nmol/L ketotifen did not modify EFS-induced contractions, while preincubation with 0.1 µmol/L or 1 µmol/L ketotifen for 1, 2 and 3 hours increased the contraction induced by EFS to a similar extent (Figure 2B–2D). Preincubation with 0.1 mmol/L or 1 mmol/L tranilast for 1 or 2 hours induced a progressive decrease in EFS-induced contractions that was stabilised after 3 hours. Preincubation with 10 µmol/L tranilast did not modify EFS-induced contractions (Figure 2E–2G). For this reason, we performed the following experiments preincubating with either 0.1 µmol/L ketotifen or 0.1 mmol/L tranilast during 3 hours.

Endothelium removal increased EFS-induced contractile response similarly in segments from all experimental groups (Figure 3A–3C). EFS-induced contractions were practically abolished in segments from all experimental groups by the blocker for nerve impulse propagation, TTX (0.1 µmol/L; table 2).

Vasodilator response to ACh

The vasodilator response to ACh was similar in all experimental groups (Figure 3D, table 3).

Effect of ketotifen or tranilast on the nitrergic component of vascular responses to EFS

Basal NO release was higher in control than in ketotifen- or tranilast-incubated mesenteric segments (Figure 4A). EFS

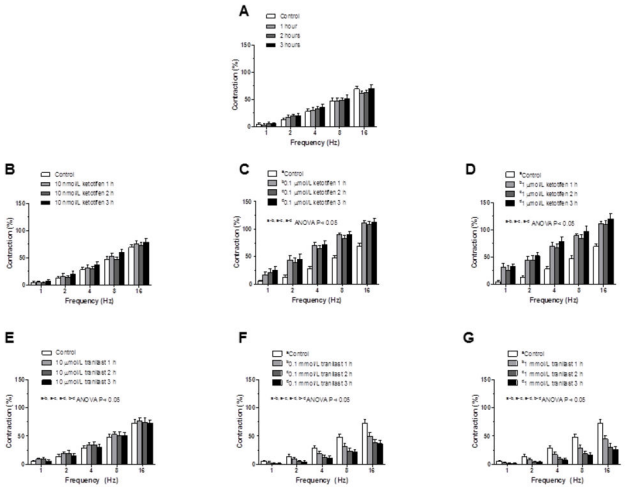

Figure 2

Figure 2. Vasoconstrictor response to EFS. (A) Isometric tension recording of the frequency-dependent contractions in intact mesenteric artery segments from Wistar rats. Effect of preincubation with 10 nmol ketotifen (B), 1 µmol/L ketotifen (C), 0.1 µmol/L ketotifen (D), 10 µmol/L tranilast (E), 0.1 mmol/L tranilast (F) or 1 mmol/L tranilast (G) for 1, 2 and 3 hours on frequency dependent contraction in mesenteric segments from Wistar rats. Results (means ± S.E.M.) are expressed as a percentage of tone induced by 75 mmol/L KCl. n= 10 animals each group.

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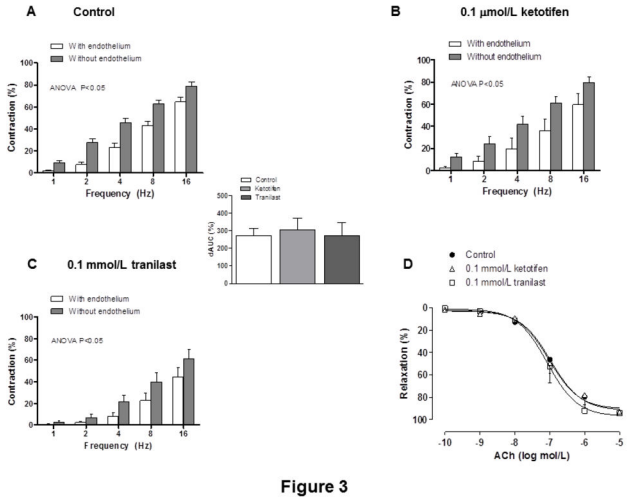

Figure 3

Figure 3. Endothelium influence on vasoconstrictor response to EFS. Effect of endothelium removal on the vasoconstrictor response to electrical field stimulation in control (A), ketotifen-incubated (B) or tranilast-incubated (C) mesenteric segments from Wistar rats. Results (means ± S.E.M.) are expressed as a percentage of tone induced by 75 mmol/L KCl. n= 10 animals each group. Insert graph shows differences of area under the curve (dAUC) in the absence or presence of 01 µmol/L phentolamine, expressed as arbitrary units. * P< 0.05 control vs. tranilast. (D) ACh-induced vasodilation in endothelium-intact control, ketotifen-incubated or tranilast-incubated mesenteric segments. Results (mean + S.E.M.) were expressed as a percentage of the previous tone elicited by exogenous NA. n = 6 animals each group.

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increased NO release in all experimental groups, but the increase was greater in control arteries (Figure 4A). Preincubation with L-NAME (0.1 mmol/L), 7NI (0.1 mmol/L) or TTX (0.1 µmol/L) practically abolished EFS-induced NO release in arteries from either treatment group (table 4). Additionally, preincubation with either the H1 receptor antagonist loratadine (1 µmol/L) or the H2 receptor antagonist famotidine (1 µmol/L) for 30 minutes did not modify basal or EFS-induced NO release (table 4).

The expression of nNOS was not modified by incubation with either ketotifen or tranilast (Figure 4B). P-nNOS expression was decreased in homogenates from ketotifen- or tranilast-incubated arteries compared to expression in control segment homogenates (Figure 4B).

In NA-precontracted mesenteric segments (Control: 995.5 ± 14.6 mg; ketotifen: 1002.9 ± 29.41 mg; tranilast: 946.7 ± 28.47 mg), DEA-NO (0.1 nmol/L–0.1 mmol/L) induced a concentration-dependent relaxation that was greater in segments preincubated with either mast cell stabilizer than in control segments (Figure 4C, table 5). The O_2^- scavenger tempol increased vasodilator response to DEA-NO in control segments, but not in ketotifen- or tranilast- incubated segments (Figure 5, table 5). Additionally, after subtracting the lucigenin chemiluminescence obtained in the presence of tiron from that obtained in its absence, the calculated tiron-quenchable chemoluminescence was significantly lower in ketotifen-incubated and tranilast-incubated segments than in control

Table 2. Effect of preincubation with tetrodotoxin (TTX, 0.1 µmol/L) or 6-hydroxydopamine (6-OHDA, 1.46 mmol/L) on the frequency–contraction curves performed in control, ketotifen-incubated (0.1 µmol/L) and tranilast-incubated (0.1 mmol/L) mesenteric segments.

	1 Hz	2 Hz	4 Hz	8 Hz	16 Hz
Control	9.2 ± 1.7	27.6 ± 3.5	45.9 ± 3.4	62.6 ± 3.3	79.1 ± 3.7
TTX	0	0	0	0.5 ± 0.04	0.9 ± 0.2
6-OHDA	0	0	0.1 ± 0.03	0.2 ± 0.05	0.4 ± 0.1
Ketotifen	17.8 ± 1.7	34.9 ± 4.1	53.5 ± 4.8	69.6 ± 3.8	90.1 ± 2.6
TTX	0	0	0	0.2 ± 0.01	0.6 ± 0.1
6-OHDA	0	0	0.2 ± 0.06	0.5 ± 0.1	0.9 ± 0.2
Tranilast	2.5 ± 1.1	7.0 ± 3.1	21.3 ± 6.1	39.9 ± 8.2	61.5 ± 8.7
TTX	0	0	0	0	0.1 ± 0.01

Results (means ± S.E.M.) are expressed as percentages of the response elicited by 75 mM KCl; zeros are used when contraction was not detected. n = 5–7 animals.

mesenteric segments. Preincubation of segments with tempol strongly decreased the tiron-quenchable chemiluminescence (Table 6). Additionally, 3-NT levels were decreased in

Table 3. E_{max} and log EC_{50} values of vasodilator responses to ACh in control, ketotifen-incubated (0.1 μmo/L) or tranilast-incubated (0.1 mmol/L) mesenteric arteries from Wistar rats.

	E_{max}	log EC_{50}
Control	91.88 ± 2.62	-6.98 ± 0.09
Ketotifen	97.22 ± 3.83	-7.07 ± 0.13
Tranilast	90.64 + 3.68	-7.02 ± 0.15

Results are expressed as means ± S.E.M. n=6 animals each group.

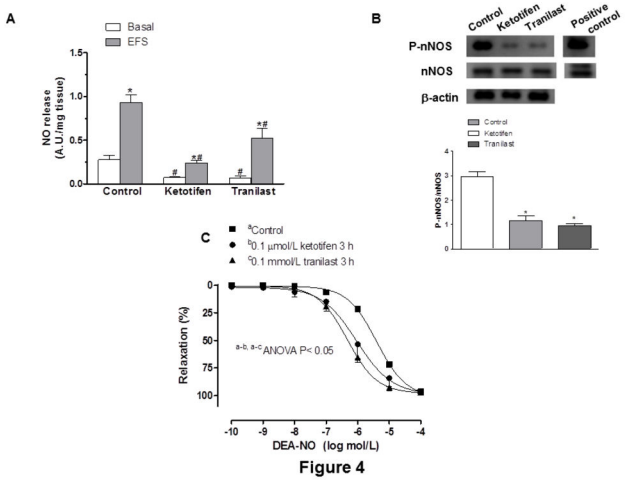

Figure 4

Figure 4. Effect of ketotifen and tranilast on neuronal NO synthesis and vasodilation (A) Effect of preincubation with ketotifen or tranilast on basal and EFS-induced NO release in mesenteric segments. n = 5 animals each group. Results (means ± S.E.M.) are expressed as arbitrary units (A.U.)/mg tissue.

*P< 0.05 vs basal; # P< 0.05 vs control. (B) Western blot for nNOS and P-nNOS expression in control, ketotifen- and tranilast-incubated mesenteric segments. Figure is representative of preparations from six samples in each group. Lower panel shows relation between densitometric analyses for P-nNOS vs. nNOS expression. * P< 0.05. (C) Vasodilator response to DEA-NO in control, ketotifen- incubated and tranilast-incubated mesenteric segments. Results (means ± S.E.M.) are expressed as a percentage of the inhibition of contraction induced by NA. n = 6 animals each group.

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ketotifen-incubated and tranilast-incubated segments (In ng 3-NT/mg protein: Control[a]: 1.71 + 0.31; 0.1 μmol/L ketotifen[b]: 0.92 + 0.06; 0.1 mmol/L tranilast[c]: 0.98 + 0.11; [a-b, a-c] P< 0.05; n = 6 animals each group).

In line with these results, the contraction induced by EFS was significantly increased by preincubation with the unspecific NOS inhibitor L-NAME (0.1 mmol/L) in control segments, but it did not have any effect in either ketotifen- or tranilast-treated segments (Figure 6). The specific iNOS inhibitor 1400W did not modify EFS-induced contraction in any experimental group (Figure 6).

Table 4. Effect of preincubation with L-NAME (0.1 mmol/L), 7-nitroindazol (7-NI, 0.1 mmol/L), loratadine 7-NI, 1 mmol/L loratadine, 1 mmol/L famotidine or 0.1 mmol/L TTX on basal and EFS-induced NO release in control, ketotifen-incubated (0.1 mmol/L) and tranilast-incubated (0.1 mmol/L) mesenteric segments.

	Basal	EFS
Control	0.28 ± 0.05	0.95 ± 0.09*
L-NAME	0.26 ± 0.04	0.29 ± 0.07#
7-NI	0.27 ± 0.09	0.31 ± 0.06#
Loratadine	0.32 ± 0.07	0.92 ± 0.13*
Famotidine	0.27 ± 0.12	0.94 ± 0.17*
TTX	0.25 ± 0.03	0.28 ± 0.11#
Ketotifen	0.07 ± 0.01	0.23 ± 0.11*
L-NAME	0.05 ± 0.03	0.07 ± 0.04#
7-NI	0.09 ± 0.04	0.11 ± 0.04#
TTX	0.06 ± 0.03	0.07 ± 0.03#
Tranilast	0.07 ± 0.02	0.38 ± 0.16*
L-NAME	0.06 ± 0.01	0.09 ± 0.04#
7-NI	0.06 ± 0.02	0.08 ± 0.03#
TTX	0.04 ± 0.01	0.04 ± 0.01#

Results (means ± S.E.M.) are expressed in arbitrary units (A.U.)/mg tissue. n = 6-10 animals each group. *P < 0.05 compared with the respective basal NO release. #P < 0.05 compared with conditions without specific inhibitor.

Table 5. E_{max} and log EC_{50} values of vasodilator responses to DEA-NO in control, ketotifen-incubated (0.1 μmol/L) or tranilast-incubated

	Untreated		Tempol-treated	
	E_{max}	log EC_{50}	E_{max}	log EC_{50}
Control	101.5 ± 2.16	-5.39 ± 0.04	94.76 ± 7.52	-6.10 ± 0.18#
Ketotifen	99.11 ± 8.40	-6.14 ± 0.18*	101.10 ± 2.38	-5.95 ± 0.15
Tranilast	98.02 ± 2.44	-6.33 ± 0.07*	98.62 ± 3.03	-6.38 ± 0.08

Results are expressed as means ± S.E.M. n= 6 animals each group. *P < 0.05 vs. control segments. #P< 0.05 tempol incubated vs. unincubated segments.

Effect of preincubation with ketotifen or tranilast on sympathetic innervation

Preincubation with 0.1 μmol/L ketotifen did not modify the NA contractile response (0.1 nmol/L–10 μmol/L) (Figure 7A), while the response was decreased in 0.1 mmol/L tranilast-preincubated segments (Figure 7A). Both basal and EFS-induced NA releases were not modified by preincubation with either 0.1 μmol/L ketotifen or 0.1 mmol/L tranilast (Table 1). The contraction elicited by EFS was significantly reduced by the α-adrenoceptor antagonist, phentolamine (1 μmol/L), in segments from control, ketotifen-incubated and tranilast-incubated segments. The decrease was lower in tranilast-incubated than in control mesenteric segments (Figure 7B–7D). Preincubation with 6-OHDA practically abolished the EFS-

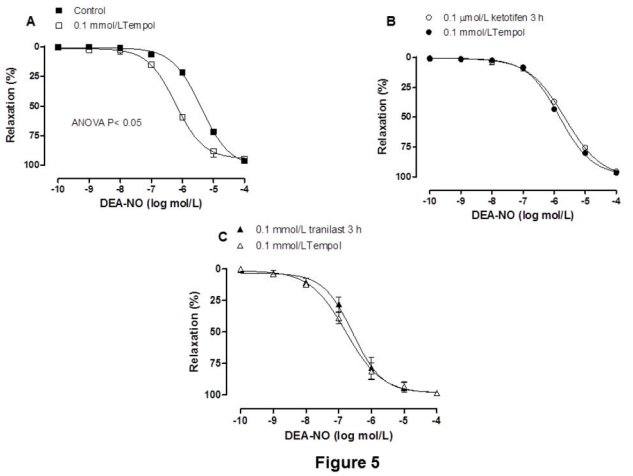

Figure 5

Figure 5. Effect of superoxide anions on NO-dependent vasodilation. Effect of 0.1 mmol/L tempol on the concentration–response curves to DEA-NO in control (A) ketotifen-incubated (B) and tranilast-incubated (C) mesenteric segments.

Results (means ± S.E.M.) are expressed as a percentage of the inhibition of the contraction induced by NA. n=6 animals each group.

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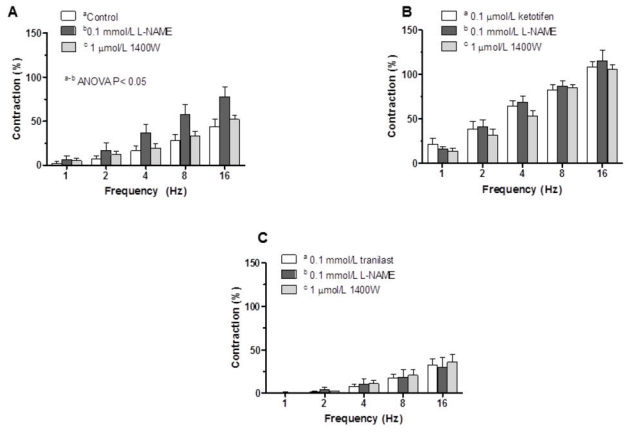

Figure 6

Figure 6. Effect of ketotifen or tranilast on nitrergic innervation function. Effect of preincubation with 0.1 mmol/L L-NAME or 1 µmol/L 1400W on the frequency-response curves in control (A) ketotifen-incubated (B) or tranilast-incubated (C) mesenteric segments. Results (means ± S.E.M.) are expressed as a percentage of tone induced by 75 mmol/L KCl. n = 6 animals each group.

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Table 6. Effect of 0.1 µmol/L ketotifen or 0.1 mmol/L tranilast on $O_2^{\cdot -}$ release. Effect of preincubation with 0.1 mmol/L tempol on $O_2^{\cdot -}$ release in control, ketotifen-incubated and tranilast-incubated mesenteric segments.

	Untreated	Tempol-treated
Control	103.4 ± 8.2	5.4 ± 1.3#
Ketotifen	54.2 ± 1.6*	2.1 ± 1.8#
Tranilast	39.1 ± 2.8*	3.1 ± 1.0#

Results (means ± S.E.M.) are expressed in chemoluminescence units/ min mg tissue. *P < 0.05 compared with control group. #P < 0.05 compared with conditions without tempol. n= 6-10 animals each group.

induced contraction in segments from control and ketotifen-incubated mesenteric segments (Table 2).

Discussion

The results of the present study demonstrate for the first time that the mast cell stabilizers ketotifen and tranilast modify EFS-induced vasoconstriction differently in rat mesenteric arteries. Ketotifen increased EFS-induced vasoconstriction, an effect that seems to be mediated at least by a decrease in neuronal NO release, while tranilast, although also decreasing neuronal NO release, diminished EFS-induced contraction through a decreased vasoconstrictor response to NA. The effect of these drugs on neuronal NO release is mediated by a decrease in nNOS phosphorylation.

The remarkable connection between innervation and mast cells has attracted much interest. This aspect has been widely studied in the abdominal cavity, where mast cell activation modulates various gastrointestinal functions [9,25–28]. In the current study, we have observed that mast cells are located in the adventitial layer, and that histamine is released under basal conditions indicating, that perivascular mast cells are tonically activated. Neurotransmitter release has been described to alter mast cell activation and their release of multiple mediators, including histamine [11,12]. In the current study, histamine release was enhanced by EFS. The fact that preincubation with TTX abolishes EFS-induced histamine release reveals for the first time that neurotransmitter release from mesenteric artery innervation activates mast cell degranulation. Both basal and EFS-induced histamine release were decreased by ketotifen or tranilast incubation, confirming the stabilizing role of these drugs.

The interplay of mast cells with perivascular neuronal function and the possible functional consequences are highly interesting when examining aspects of hemodynamic changes when mast cells are stabilized. Thus, the current study was designed to analyze the effect of mast cell stabilization on perivascular innervationfunction. For this purpose, we studied the effect of ketotifen and tranilast on the vasomotor response produced by EFS in rat superior mesenteric arteries.

We first analyzed whether ketotifen and tranilast modified the EFS-induced contraction observed in mesenteric segments. As we showed in the *Results* section, after performing a time-course pilot study, we considered it appropriate to perform experiments with either 0.1 µmol/L ketotifen or 0.1 mmol/L tranilast for 3 hours. EFS produced a frequency-dependent

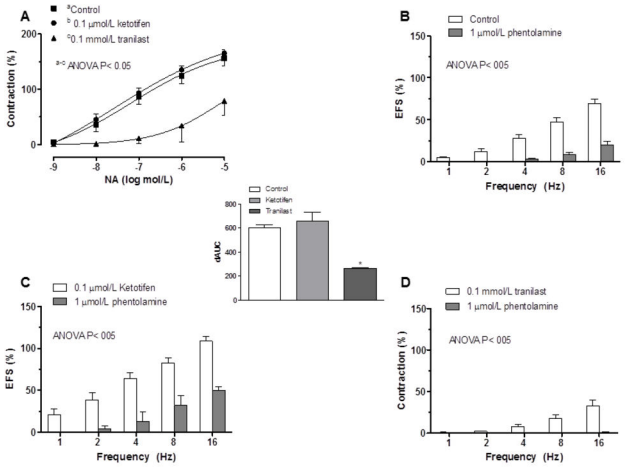

Figure 7

Figure 7. Effect of ketotifen or tranilast on sympathetic innervation function. (A) Effect of preincubation with ketotifen or tranilast on the vasocostrictor response curves to noradrenaline. Effect of 0.1 µmol/L phentolamine on the frequency-response curve in control (B), ketotifen-incubated (C) or tranilast-incubated (D) segments. Results (means ± S.E.M.) are expressed as a percentage of tone induced by 75 mmol/L KCl.

n = 5-6 animals each group. Insert graph shows differences of area under the curve (dAUC) in the absence or presence of 01 µmol/L phentolamine, expressed as arbitrary units. *** P< 0.05 control vs. tranilast**.

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contraction in endothelium-intact mesenteric segments from all the experimental groups as reported in previous reports by our group [22,23,29,30]. We observed that ketotifen and tranilast had opposite effects: while preincubation with ketotifen increased the contraction induced by EFS, tranilast decreased that contraction. These modifications were not attributable to changes in the intrinsic contractile machinery as was demonstrated by the similar vasoconstrictor response to KCl in all experimental groups. Endothelium removal increased vasoconstrictor response to EFS to the same extent in the three experimental groups, indicating that the modulating role of endothelium is not modified by either ketotifen or tranilast. The fact that ACh-induced vasodilation was not modified in any experimental group reinforces this observation. Therefore, these results indicate that the modifications observed after preincubation with either ketotifen or tranilast are due to modifications in perivascular innervation function, as was confirmed by the abolishment of EFS-induced vasoconstriction in the presence of TTX.

Superior mesenteric artery possesses different innervations, mainly vasodilator nitrergic and vasoconstrictor sympathetic innervations, which are implicated in the control of vascular tone [6–8]. We analyzed the participation of each kind of innervation in the vasoconstriction induced by EFS, and the possible modifications in this participation that were induced by either ketotifen or tranilast.

Ketotifen and tranilast have been reported to alter eNOS and iNOS activity in several tissues [31–33]. However, to our knowledge, there are no reports about possible alterations in nNOS expression and/or activity in perivascular nerve endings. Therefore, our next objective was to analyze the possible effect of ketotifen and tranilast on neuronal NO release from nitrergic nerve endings. Preincubation with ketotifen or tranilast diminished both basal and EFS-induced NO release. The fact that, in all cases, EFS-induced NO release was abolished by preincubation with TTX, L-NAME, the non-specific NOS inhibitor, or 7-nitroindazol (7-NI), the specific nNOS inhibitor, confirms the neural origin of the NO release.

Besides its role as a mast cell membrane stabilizer, it has been clearly demonstrated that ketotifen also possesses powerful and sustained non-competitive histamine blocking properties, as it antagonizes both the H1 and H2 histamine receptors. The fact that neither the selective H1 antagonist loratadine nor the selective H2 antagonist famotidine modified either basal or EFS-induced NO release rules out the participation of histamine receptors in this response.

NO released from nerve endings is biosynthesized by nNOS [8,22,23,30]. Since preincubation with either ketotifen or tranilast decreased both basal and EFS-induced NO release, our next objective was to determine if these decreases produced by ketotifen and tranilast were due to modifications in nNOS expression and/or activity. We found that nNOS protein expression was not modified. nNOS must be phosphorylated in order to be activated. P-nNOS expression was decreased by treatment with either ketotifen or tranilast. These results indicate that the decreased NO release observed after preincubation with either ketotifen or tranilast is due to a decrease in nNOS activation. To our knowledge this is the first study to demonstrate the actions of ketotifen and tranilast on neuronal NO release from perivascular innervation in mesenteric bed.

Previously, we have demonstrated in this rat strain that both the non-selective NOS inhibitor L-NAME and the specific nNOS inhibitor 7-NI decrease EFS-induced NO release to a similar extent [30]. However, in vascular reactivity experiments, preincubation with 7-NI also decreased vasoconstrictor response to NA, making the analysis of EFS-induced contractions very complex, and leading to result misinterpretation [29,30]. For that reason, we used L-NAME in vascular reactivity experiments. L-NAME did not modify EFS-induced contractions in ketotifen or tranilast-incubated segments, in contrast to the increase observed in control segments, thus suggesting that both ketotifen and tranilast decrease nitrergic innervation function. Preincubation with the specific iNOS inhibitor 1400W did not modify vasoconstrictor response to EFS, ruling out an iNOS participation in the effects of ketotifen and tranilast.

Possible alterations in smooth muscle sensitivity to NO cannot be ruled out. A decrease in O_2^- was observed in ketotifen- and tranilast-incubated mesenteric segments, in agreement with the previously described antioxidant effect of both mast cell stabilizers [34,35]. Additionally, 3-NT detection, used as a stable marker of peroxynitrite detection [36], showed a marked decrease in segments preincubated with either

ketotifen or tranilast. Therefore, both ketotifen and tranilast could alter NO metabolism and bioavailability. The vasodilator response to DEA-NO was increased by preincubation with either ketotifen or tranilast. Preincubation with the O_2^-scavenger tempol increased vasodilator response to DEA-NO in control segments, but not in ketotifen or tranilast-incubated segments, thus confirming a decreased NO metabolism through decreased O_2^-release.

Therefore, ketotifen and tranilast induce two opposite effects: a decrease in neuronal NO release, and an increase in NO bioavailability due to a decrease in O_2^- formation. The net effect is a decreased role for nitrergic innervation.

The fact that both ketotifen and tranilast exert the same effect on neuronal NO release does not explain why they produce opposite effects in the EFS-induced contractile response. Therefore, different influences by ketotifen and tranilast on the function of other innervations cannot be ruled out. Given the principal role played by sympathetic innervation in EFS-induced vasoconstriction [2,8], we analyzed the possible influence of ketotifen and tranilast on EFS-induced vasoconstriction. Vasoconstriction elicited by EFS was strongly reduced by phentolamine in segments from all experimental groups, indicating that this response was mediated mainly by NA release from the adrenergic component of sympathetic nerve terminals, with subsequent activation of α-adrenoceptors. This decrease was similar in control and ketotifen-incubated mesenteric segments. However, the decrease in EFS-induced contraction obtained by preincubation with phentolamine was lower in tranilast-preincubated segments, suggesting different influences by ketotifen and tranilast on the adrenergic component of sympathetic innervation. These differences could be due to modifications in either NA release or vasoconstrictor response to exogenous NA. When analysing NA release, we observed that it was not modified by mast cell stabilization with either ketotifen or tranilast. This result shows that the effect of these drugs on EFS-induced vasoconstriction is not mediated by changes in NA release. When concentration–response curves to exogenous NA were performed, we observed that ketotifen did not modify the vasoconstrictor response, while tranilast decreased it. Taken together, these results confirm that the adrenergic component of sympathetic innervation is not affected by ketotifen in the mesenteric artery, as also reported in other tissues [37]. However, although tranilast did not affect NA release, it did reduce the contractile response, as observed with other vasoconstrictor agents [18,38]. This effect can be attributed to inhibition of the Ca^{2+} influx from the extracellular environment and Ca^{2+} release from intracellular Ca^{2+} stores, as previously reported [39,40]. The fact that vasoconstriction produced by KCl remained unmodified in the presence of tranilast suggests that this drug interferes with the Ca^{2+} movement coupled to receptor activation, but not to the movement induced by depolarization. This differential influence on adrenergic innervation seems to be implicated in the opposite results obtained when incubating with one mast cell stabilizer or the other.

After preincubation with phentolamine, we observed a remnant contractile response to EFS in control and ketotifen-incubated segments, but not after tranilast treatment. This result indicates that tranilast abolished the function of another vasoconstrictor factor. The depletion of sympathetic innervation in control and ketotifen treated segments by preincubation with the neurotoxin 6-OHDA abolished the remant vasoconstriction, thereby confirming that this contractile neurotransmitter has a sympathetic origin in control and ketotifen-incubated segments. This neurotransmitter could probably be ATP, as we have previously observed in other experimental conditions [21,30].

Taken together, these results indicate that both ketotifen and tranilast alter the vasoconstrictor response to EFS in rat mesenteric artery, but in an opposite manner: ketotifen increases EFS-induced contraction, while tranilast decreases it. This fact is due to different actions by these drugs on the sympathetic and nitrergic innervations: both ketotifen and tranilast diminish nitrergic innervation function through a decrease in nNOS activation, while tranilast also decreases sympathetic innervation function, mainly through decreased NA vasoconstriction. These results indicate that the election of the mast cell stabilizer could be relevant, since it could induce important hemodynamic changes.

Author Contributions

Conceived and designed the experiments: GB JBR. Performed the experiments: ES LCL JBR. Analyzed the data: ES LCL FEX GB JBR. Contributed reagents/materials/analysis tools: GB FEX. Wrote the manuscript: ES LCL FEX GB JBR.

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Artículo 9: Tranilast increases vasodilator response to acetylcholine in rat mesenteric resistance arteries through increased EDHF participation.

Xavier FE, Blanco-Rivero J, **Sastre E**, Caracuel L, Callejo M, Balfagón G.

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Tranilast Increases Vasodilator Response to Acetylcholine in Rat Mesenteric Resistance Arteries through Increased EDHF Participation

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Abstract

Background and Purpose: Tranilast, in addition to its capacity to inhibit mast cell degranulation, has other biological effects, including inhibition of reactive oxygen species, cytokines, leukotrienes and prostaglandin release. In the current study, we analyzed whether tranilast could alter endothelial function in rat mesenteric resistance arteries (MRA).

Experimental Approach: Acetylcholine-induced relaxation was analyzed in MRA (untreated and 1-hour tranilast treatment) from 6 month-old Wistar rats. To assess the possible participation of endothelial nitric oxide or prostanoids, acetylcholine-induced relaxation was analyzed in the presence of L-NAME or indomethacin. The participation of endothelium-derived hyperpolarizing factor (EDHF) in acetylcholine-induced response was analyzed by preincubation with TRAM-34 plus apamin or by precontraction with a high K^+ solution. Nitric oxide (NO) and superoxide anion levels were measured, as well as vasomotor responses to NO donor DEA-NO and to large conductance calcium-activated potassium channel opener NS1619.

Key Results: Acetylcholine-induced relaxation was greater in tranilast-incubated MRA. Acetylcholine-induced vasodilation was decreased by L-NAME in a similar manner in both experimental groups. Indomethacin did not modify vasodilation. Preincubation with a high K^+ solution or TRAM-34 plus apamin reduced the vasodilation to ACh more markedly in tranilast-incubated segments. NO and superoxide anion production, and vasodilator responses to DEA-NO or NS1619 remained unmodified in the presence of tranilast.

Conclusions and Implications: Tranilast increased the endothelium-dependent relaxation to acetylcholine in rat MRA. This effect is independent of the nitric oxide and cyclooxygenase pathways but involves EDHF, and is mediated by an increased role of small conductance calcium-activated K+ channels.

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☯ These authors contributed equally to this work.

Introduction

Mesenteric blood flow can constitute up to 20–30% of total cardiac output [1] and is regulated by different mechanisms in which endothelial factors like nitric oxide (NO), prostanoids and endothelium-derived hyperpolarizing factor (EDHF) play a pivotal role. Modifications in the release and/or participation of these vasoactive substances can alter peripheral vascular resistance, with the role of resistance vessels being especially relevant.

Mast cells play an important role in several physiological and pathological situations such as intestinal motility, angiogenesis and atherosclerosis [2–4]. When activated, mast cells secrete numerous vasoactive and proinflammatory mediators, such as histamine, serotonin, bradykinin, endothelin, NO, leukotrienes, prostaglandins, or cytokines [5], which could alter vascular endothelial and smooth muscle function [6]. These consequences are highly interesting, particularly aspects of hemodynamic changes when mast cells are stabilized. Tranilast was initially used to treat allergic diseases due to its capacity to inhibit mast cell degranulation [7] and has also been suggested in the treatment of multiple inflammatory processes, including various pathologies where blood flow is altered, such as in the vasodilation induced by allergic processes [8–11].

Previously our group has described that lipopolysaccharide, a model of endotoxic shock, influences vascular tone by modifying both endothelial and neuronal factors [12,13]. Additionally, we have studied the effect of tranilast on the vasoconstrictor response produced by electrical field stimulation (EFS) in rat superior mesenteric arteries, demonstrating that it diminished the vaso-

constrictor response to EFS by decreasing noradrenaline-induced vasoconstriction [14] although it did not influence endothelial function in this artery, as similarly reported by Yang et al [15] in rat aorta. However, mesenteric resistance arteries play a pivotal role in the regulation of vascular resistance, and differences in endothelial function have been previously described in different vascular beds under the same experimental conditions [16,17]. With this in mind, the possible effect of tranilast on endothelial function in resistance vessels may help induce hemodynamic changes that could be relevant in the treatment of pathologies like allergy.

Since total peripheral resistance mainly depends on resistance vessels, and the role that mesenteric resistance arteries play in this is very relevant, we consider it very important to analyze the possible alterations tranilast may produce in the endothelial function of these vessels.

Materials and Methods

Ethics Statement

All animals were housed in the Animal Facility of the Universidad Autónoma de Madrid (Registration number EX-021U) in accordance with directive 609/86 of the E.E.C., R.D. 233/88 of the Ministerio de Agricultura, Pesca y Alimentación of Spain, and Guide for the Care and Use of Laboratory Animals published by the USA National Institutes of Health [NIH publication No. 85.23, revised 1985]. The experimental protocol was approved by the Ethics Committee of the Universidad Autónoma de Madrid.

Animals

We used 6 month-old male Wistar rats. Rats were sacrificed by CO_2 inhalation followed by decapitation; the mesenteric vascular bed was removed and placed in cold (4°C) Krebs-Henseleit solution (KHS; in mmol/L: 115 NaCl, 2.5 $CaCl_2$, 4.6 KCl, 1.2 KH_2PO_4, 1.2, $MgSO_4.7H_2O$, 25 $NaHCO_3$, 11.1 glucose, and 0.03 EDTA).

Perivascular mast cell detection

The third-order branches from mesenteric resistance arteries were fixed in 4% formaldehyde in phosphate buffered saline solution (PBS, pH = 7.4) for 1 hour, cryoprotected with 30%w/v sucrose in PBS (overnight), transferred to a cryomold containing Tissue-Tek OCT embedding medium (20 min) and then immediately frozen in liquid nitrogen. All samples were kept at −70°C until the day of the experiments. Frozen tissue segments were cut into 10 μm thick sections, placed on glass slides and stained with 0.1% Toluidine Blue (3 min) for perivascular mast cell detection, as previously described [14]. Sections were coverslipped and light microscopy images were taken (Nikon Eclipse TE2000-S [inverted microscope], Nikon DXM1200F [digital camera]).

Vascular reactivity study

For reactivity experiments the third-order branch of the mesenteric arcade was dissected and cut in segments of approximately 2 mm in length. Segments of mesenteric resistance arteries were mounted in a small vessel chamber myograph (Danish Myo Technology A/S, Äarhus, Denmark) to measure isometric tension according to the method described by Mulvany and Halpern [18]. After a 15-min equilibration period in oxygenated KHS at 37°C and pH 7.4, segments were stretched to their optimal lumen diameter for active tension development. Optimal lumen diameter was determined based on the internal circumference/wall tension ratio of the segments by setting the internal circumference, L_0, to 90% of what the vessels would have if they were exposed to a passive tension equivalent to that produced by a transmural pressure of 100 mmHg [18]. Optimal lumen diameter was determined using specific software for normalization of resistance arteries (DMT Normalization Module; ADInstruments Pty Ltd, Castle Hill, Australia). Segments were washed with KHS and left to equilibrate for 30 min. Vessel contractility was then tested by an initial exposure to a high-K^+ (120 mmol/L) solution.

After washout, segments were contracted with a concentration of noradrenaline that induced approximately 50%–70% of the maximum contraction elicited by KCl, and then acetylcholine (1 μmol/L) was added to assess the integrity of the endothelium. Some segments were subjected to mechanical endothelium removal. The absence of endothelium was confirmed by the inability of acetylcholine (1 μmol/L) to induce relaxation. Endothelium removal did not modify KCl- (120 mmol/L) induced contraction.

Since the level of smooth muscle constriction can itself antagonize the extent of the endothelium-dependent relaxation, we performed the following experiments adjusting the dose of NA or KCl to a concentration which allowed us to reach a 50–70% of the maximum contraction elicited by KCl.

Experimental protocols

The segments were rinsed with KHS for 1 h and then a cumulative concentration-response curve to ACh (0.1 nmol/L to 3 μmol/L) was obtained in noradrenaline-precontracted segments preincubated or not with tranilast (100 μmol/L, 1 hour, time and dose obtained from previous pilot studies). The concentration of tranilast used and the time of incubation were from previous pilot studies, performed similarly to our previous study [14]. Additionally, vasoconstrictor responses to alpha-adrenergic agonist noradrenaline (10 nmol/L to 0.1 mmol/L) were performed in both control and tranilast-incubated segments.

The possible role of NO in ACh-induced relaxation was investigated in tranilast-treated and untreated segments by preincubation with 100 μmol/L L-NAME (a non-selective nitric oxide synthesis inhibitor) before performing concentration-response curves to ACh. Additionally, endothelium-independent relaxation was studied by evaluating relaxation to NO donor DEA-NO (10 nmol/L to 300 μmol/L) in arteries previously contracted with noradrenaline.

The role of EDHF in the ACh-induced relaxation was analyzed. For this purpose, the vasodilator response to ACh in segments precontracted high K^+ solution (at a concentration that produced approximately 50–70% of the contraction induced by 120 mM KCl) was studied. Additionally, the effect of a calcium-activated potassium channel blockade, produced by apamin (1 μmol/L) plus TRAM-34 (0.1 μmol/L), on the ACh response was analyzed in NA-precontracted arteries pretreated or not with tranilast. In another set of experiments, the effect of L-NAME plus TRAM-34 plus apamin on ACh-induced relaxation was studied. To determine whether tranilast modified the participation of each potassium channel individually, concentration response curves to acetylcholine were performed in the presence of L-NAME plus apamin or L-NAME plus TRAM-34. All drugs were added 30 min before the concentration-response curve to ACh. Additionally, to rule out an effect of tranilast on NO mediated hyperpolarization, concentration-response curves to DEA-NO were performed in control and tranilast-incubated mesenteric segments precontracted with a high K^+ solution.

The effect of tranilast on the smooth muscle calcium-activated potassium channels was analyzed. For this purpose, the relaxation

produced by NS1619 (10 nmol/L-100 μmol/L), a large conductance calcium-activated potassium channel opener, was analyzed in NA-precontracted endothelium-denuded arteries preincubated or not with tranilast.

The participation of COX-derived metabolites was investigated in tranilast-treated and untreated segments. Arteries were preincubated with the non-specific COX inhibitor indomethacin (10 μmol/L) before performing concentration-response curves to ACh.

Nitric Oxide release

Nitric oxide release was determined using the fluorescent probe 4,5-diaminofluorescein (DAF-2), as previously described [19]. Briefly, the second, third and fourth branches of mesenteric artery were divided in two experimental groups: control and tranilast-incubated segments (100 μmol/L, 1 hour). After an equilibration period of 30 min in HEPES (in mmol/L: 119 NaCl, 20 HEPES, 46 KCl, 1 $MgSO_4.7H_2O$, 0.15 $Na_2HPO_4.12H_2O$, 0.4 KH_2PO_4, 5 $NaHCO_3$, 1.2 $CaCl_2.2H_2O$, 5.2 glucose) at 37°C, arteries were incubated with 2 μmol/L DAF-2 for 45 min and medium was collected to measure basal NO release. Once the organ bath was refilled, ACh-induced NO release was measured after an ACh concentration-curve (0.1 nmol/L - 3 μmol/L) was applied at 2-min intervals each dose. The fluorescence of the medium was measured at room temperature using a spectrofluorimeter (LS50 Perkin Elmer Instruments, FL WINLAB Software) with excitation wavelength set at 492 nm and emission wavelength at 515 nm. The stimulated NO release was calculated by subtracting the basal NO release from that evoked by ACh. Also, blank measurement samples were collected from medium without mesenteric segments in order to subtract background emission. Some assays were performed in the presence of L-NAME in order to assure assay specificity. The amount of NO released was expressed as arbitrary units/mg tissue.

Detection of superoxide anions

Superoxide anions levels were measured using lucigenin chemiluminescence, as previously described [20]. Briefly, the second, third and fourth branches of mesenteric artery, divided in two experimental groups, control and tranilast-incubated segments (100 μmol/L, 1 hour), were equilibrated for 30 min in HEPES buffer at 37°C, transferred to test tubes that contained 1 mL HEPES buffer (pH 7.4) containing lucigenin (5 μmol/L) and then kept at 37°C. The luminometer was set to report arbitrary units of emitted light; repeated measurements were collected during 5 min at 10 s intervals and averaged. 4,5-dihydroxy-1,3-benzene-disulphonic acid "Tiron" (10 mmol/L), a cell permeant, non-enzymatic superoxide anion scavenger, was added to quench the superoxide anion-dependent chemiluminescence. Also, blank samples were collected in the same way without mesenteric segments to subtract background emission.

Drugs

Drugs used were tranilast, atropine, noradrenaline hydrochloride, acetylcholine chloride, DEA-NO, indomethacin, apamin, tiron, TRAM-34, NS1619 (Sigma; St. Louis, MO, U.S.A.). Stock solutions of acetylcholine, apamin, tiron, TRAM-34 and DEA-NO were made in distilled water, noradrenaline was dissolved in a NaCl (0.9%)-ascorbic acid (0.01% wv-1) solution; indomethacin was dissolved in ethanol;tranilast, NS1619 was dissolved in dimethyl sulfoxide. These solutions were kept at −20°C and appropriate dilutions were made on the day of the experiment.

Statistical analysis

Contractions to noradrenaline were expressed as the percentage of contraction of the maximum contractile response induced by a previous contraction of KCl. Relaxation to ACh, DEA-NO and NS1619 were expressed as a percentage of the level of preconstriction induced by noradrenaline or KCl. For each concentration-response curve the maximum effect (Emax) and the concentration of agonist that produced half of the Emax (log EC50) were calculated using non-linear regression analysis (GraphPad Prism Software, San Diego, CA). The sensitivity of the agonists is expressed as pD2 (−log EC50).

All values are expressed as means ± S.E.M. of the number of animals used in each experiment. Statistical analysis was done by comparing the curve obtained in the presence of the different substances with the control curve by means of a non-repeated measure analysis of variance (ANOVA) followed by the Bonferroni post-hoc test. Some results were expressed as differences of area under the curve (dAUC). AUC were calculated from the individual concentration-response plots. For dAUC, NO and superoxide anion release experiments, the statistical analysis was done using one-way ANOVA followed by Newman-Keuls post-hoc test. $P<0.05$ was considered significant.

Results

Mast cells were detected in the adventitial layer of mesenteric arteries using toluidine blue staining (Figure 1).

Preincubation with 100 μmol/L tranilast did not modify vasoconstrictor response to 120 mmol/L KCl (Control: 14.5±1.5 mN; Tranilast: 15.1±1.3 mN/mm; $p>0.05$), while it shifted the noradrenaline-induced contractile curve to the right (Figure 2A). Cumulative addition of ACh evoked endothelium-dependent relaxations in noradrenaline-contracted arteries. 10 μmol/L and 1 μmol/L tranilast concentrations did not produce any modification on ACh-induced vasodilation in 1–3 hours incubations (Results not shown), while 1 hour-preincubation with 100 μmol/L tranilast shifted the concentration response curve to ACh to the left (Figure 2B and Table 1).

NO synthase inhibitor L-NAME decreased ACh-induced relaxation to a similar extent in both control and tranilast-incubated mesenteric segments (Figures 3A and 3B, Table 1). Relaxation to DEA-NO was not changed by tranilast either in NA-precontracted or in KCl-precontracted mesenteric arteries (Figures 3C, Table 2). In line with this, both basal and ACh-

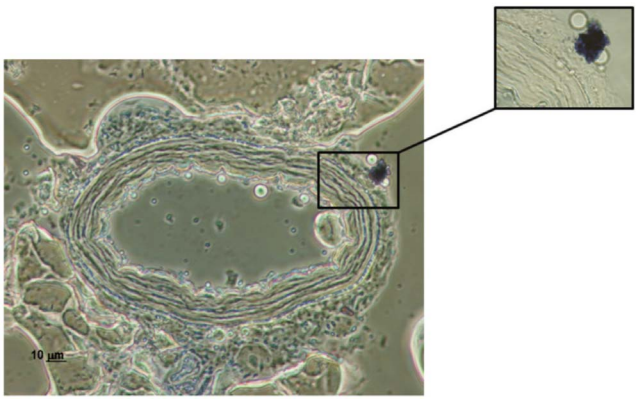

Figure 1. Mast cell localization by toluidine blue staining. Figure is representative of preparations from four rats. Magnification: 400X (general vision) and 600X (inset).
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Table 1. Effect of indomethacin, L-NAME, Apamin plus TRAM-34 or L-NAME plus Apamin plus TRAM-34 on E_{max} and pD2 to acetylcholine in untreated and tranilast-treated MRA.

	Untreated		Tranilast-treated	
	E_{max}	pD_2	E_{max}	pD_2
Control	87.6±2.03	7.43±0.04	93.5±1.64	8.12±0.04^{+}
Indomethacin	85.5±2.51	7.44±0.06	97.1±5.83	8.11±0.13^{+}
L-NAME	71.4±3.09*	7.27±0.07	87.1±2.18^{+}	7.69±0.11*$^{+}$
Apamin+TRAM-34	43.5±5.55*	7.29±0.12	46.6.5±2.82*	7.47±0.11*
L-NAME+Apamin+TRAM-34	4.33±2.60*	-	3.83±2.21*	-

Values represent means ± S.E.M.
*P<0.05 *vs.* situation without specific drugs;
$^{+}$P<0.05, Tranilast-treated *vs.* untreated.
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stimulated NO releases were similar in tranilast-treated and untreated mesenteric resistance arteries (Figure 3D). Preincubation with L-NAME abolished NO release in all experimental groups (results not shown). Superoxide anion release was similar in both tranilast-treated and untreated segments (In chemiluminiscence units/min mg tissue: Control: 10.92±3.5; Tranilast: 12.03±3.7; P<0.05.).

The concentration response curve to ACh was shifted to the right in KCl-precontracted segments after preincubation with 100 μmol/L tranilast (Figure 4A and 4B). Similarly, preincubation with apamin plus TRAM-34 shifted the ACh-induced relaxation leftward to a greater extent in tranilast-incubated segments than in control segments (Figure 4C and 4D). Combined preincubation with L-NAME plus TRAM-34 reduced ACh-induced relaxation similarly in both control and tranilast-incubated segments. However, preincubation with both L-NAME and apamin shifted the ACh-induced relaxation to the left more markedly in tranilast-incubated segments. The remnant vasodilation observed after preincubation with L-NAME plus TRAM-34 was higher in tranilast-incubated compared to control segments, while it was similar in both experimental conditions after preincubation with L-NAME plus apamin. (Figure 5). Vasodilator response to NS1619 remained unmodified in presence of tranilast. (Figure 6).

In tranilast-treated and untreated segments, ACh-induced vasodilation was not modified by indomethacin (Figure 7, Table 1). In line with this, the combined inhibition of NO and EDHF through preincubation with L-NAME plus apamin plus TRAM-34 abolished the increase in relaxation to ACh produced by tranilast (Figure 7, Table 1).

Discussion

The present results show that tranilast increased the endothelium-dependent relaxation to ACh in rat mesenteric resistance arteries. This effect is independent of the NO or COX pathways and seems to be mediated by an increase in EDHF contribution.

Under physiological conditions, mast cells have been identified in several locations in the mesentery, including around the mesenteric vessels [14,21]. When activated, mast cells secrete numerous vasoactive and proinflammatory mediators, such as histamine, serotonin, bradykinin, endothelin, NO, leukotrienes, prostaglandins, or cytokines [5], which could alter vascular endothelial and smooth muscle function [22]. Tranilast is a mast cell stabilizer used in various pathologies where blood flow is altered [8–11], such as allergy, which produces an intense vasodilation produced by histamine release from mast cells. In this study we have located perivascular mast cells around mesenteric resistance vessels, as has been described in superior mesenteric artery [14]. Previously, we have described that tranilast decreases EFS-induced vasoconstriction in superior mesenteric arteries [14]. Since total peripheral resistance mainly depends on

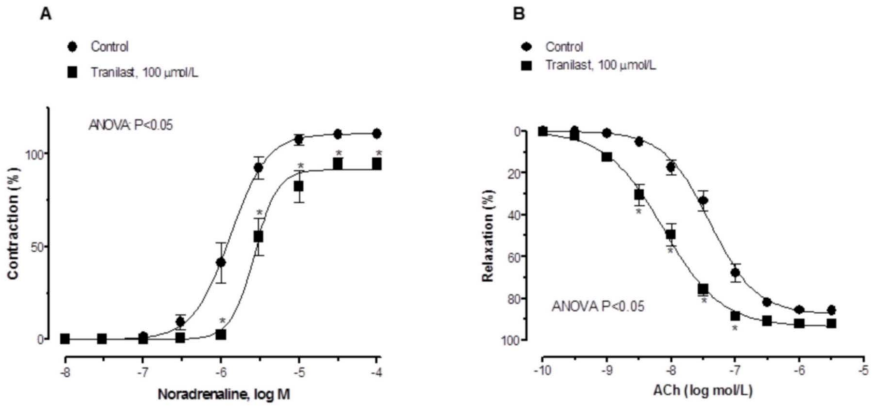

Figure 2. Effect of tranilast on endothelial function. NA-induced vasoconstriction in control and tranilast-treated mesenteric resistance arteries (A).Endothelium-dependent relaxation induced by ACh in NA-precontracted control and tranilast-treated rat resistance arteries (B) Results are expressed as mean ± S.E.M. *P<0.05 control vs. tranilast. N = 6–7 animals each group.
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Table 2. E_{max} and pD2 values of DEA-NO in untreated and tranilast-treated MRA.

	Untreated		Tranilast-treated	
	E_{max}	pD_2	E_{max}	pD_2
NA-precontracted	96.95±5.12	5.78±0.13	94.25±4.46	5.73±0.10
KCl-precontracted	70.89±3.31*	5.87±0.23	72.97±4.07*	5.61±0.12

Values represent means ± S.E.M.
*P<0.05 KCl precontraction *vs.* NA precontraction.
*P<0.05 NA-precontracted vs. KCl precontracted.
doi:10.1371/journal.pone.0100356.t002

resistance vessels, and the role that mesenteric resistance arteries play in this is very relevant, we consider it very important to analyze the possible alterations tranilast may produce in the endothelial function of these vessels. When analyzing endothelium-dependent relaxation induced by ACh in mesenteric resistance arteries, we observed an increase in this vasodilator response in segments preincubated with tranilast. Similar changes in the endothelial function observed in several pathologic situations in these vessels are associated to decreased vascular resistance and subsequent hemodynamic changes [19,23]. This outcome contrasts with previous studies, in which ACh-induced vasodilation was not modified by tranilast in superior mesenteric artery or aorta, despite the longer treatment period used [14,15]. These results indicate that tranilast can modify endothelial factor release and/or sensitivity differentially depending on the vascular bed analyzed, which is not surprising since we have previously described a similar effect in these vascular beds [24], probably associated to differences in the composition of endothelial factors.

Previous studies show that endothelial dysfunction is related to an increase in the vasoconstrictor responses to different agonists and vice versa [20,25,26]. In our experimental conditions, vasoconstriction produced by KCl remained unmodified in the presence of tranilast, similar to observations in superior mesenteric arteries [14], suggesting that this drug does not modify vascular contractile capacity. Additionally, when analyzing the vasoconstrictor response to the alpha-adrenergic agonist noradrenaline, we observed that response was decreased in tranilast-incubated segments, similarly to descriptions in superior mesenteric artery [14], but in contrast to observations in rat aorta [15].

The relaxation evoked by ACh is mediated, depending on the vascular bed analyzed, by the release of endothelium-dependent relaxing factors such as NO, prostacyclin, and EDHF [27–29]. In

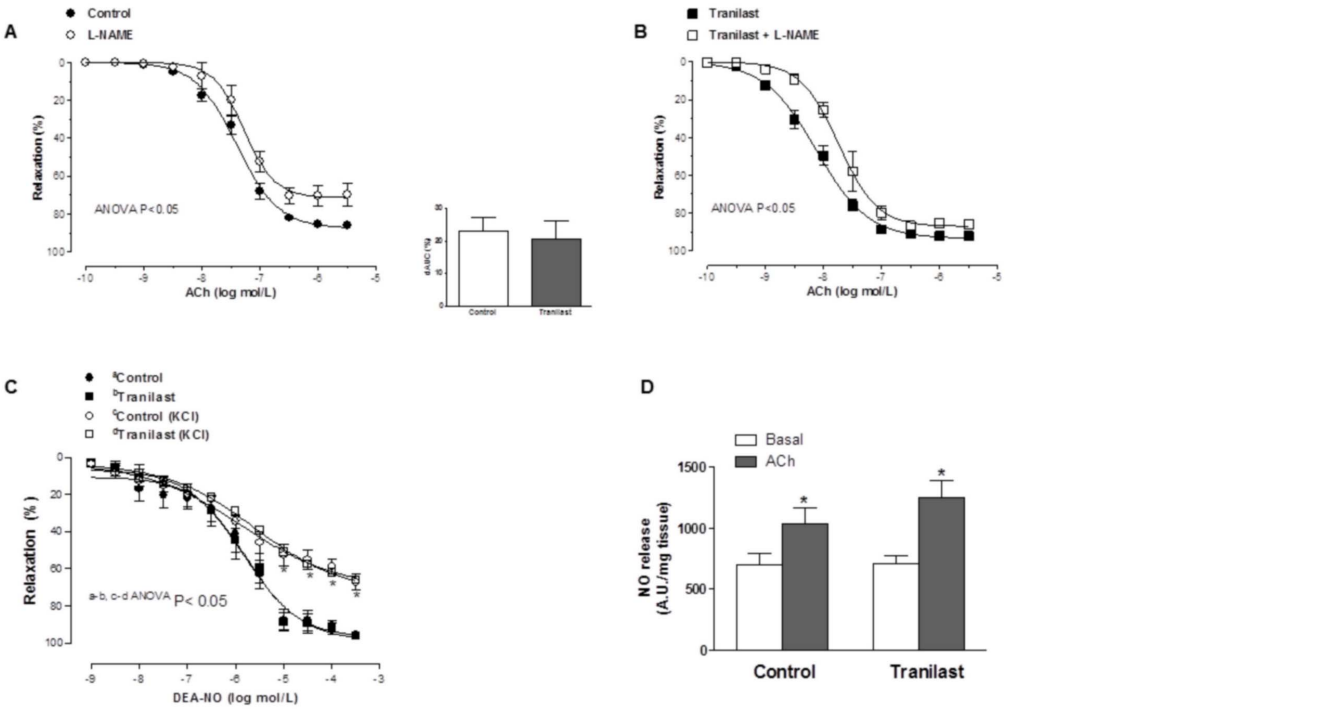

Figure 3. Participation of NO on the vasodilator response to acetylcholine. Effect of L-NAME (100 μM) on the concentration-dependent relaxation to ACh in control (A) and tranilast-treated (B) mesenteric resistance arteries. Insert graph shows the differences of area under the curve (dAUC) in control and tranilast-treated arteries pre-treated with L-NAME. Results are expressed as mean ± SEM. N = 6–7 animals in each group. (C) Vasodilator response to DEA-NO in control and tranilast-incubated mesenteric resistance arteries, precontracted with either noradrenaline or KCl. Results are expressed as mean ± S.E.M. N = 5–6 animals each group. (D) Effect of tranilast on basal and acetylcholine-induced NO release in rat mesenteric resistance arteries. Results (mean ± S.E.M.) are expressed as arbitrary fluorescence units (A.U.)/mg tissue. N = 4 animals each group. *P< 0.05 vs. basal.
doi:10.1371/journal.pone.0100356.g003

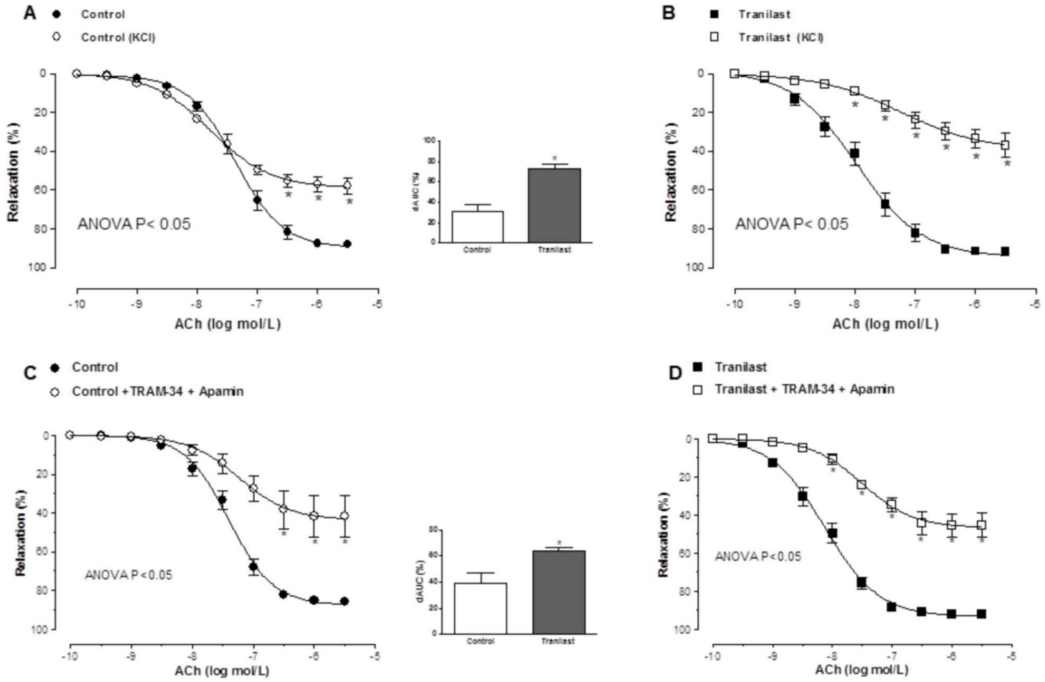

Figure 4. Participation of EDHF in the vasodilator response to acetylcholine. Relaxation to acetylcholine in control (A) and tranilast-treated arteries (B) pre-contracted with KCl. Effect of preincubation with 1 μM apamin plus 0.1 μM TRAM-34 on endothelium-dependent relaxation to acetylcholine in noradrenaline-pre-contracted control (C) and tranilast-treated arteries (D). Insert graph shows the differences of area under the curve (dAUC) in control and tranilast-treated arteries either preconstricted with KCl or pre-treated with TRAM-34 plus Apamin. Results are expressed as mean ± SEM. *P<0.05 control vs. tranilast. N = 5–7 animals in each group.
doi:10.1371/journal.pone.0100356.g004

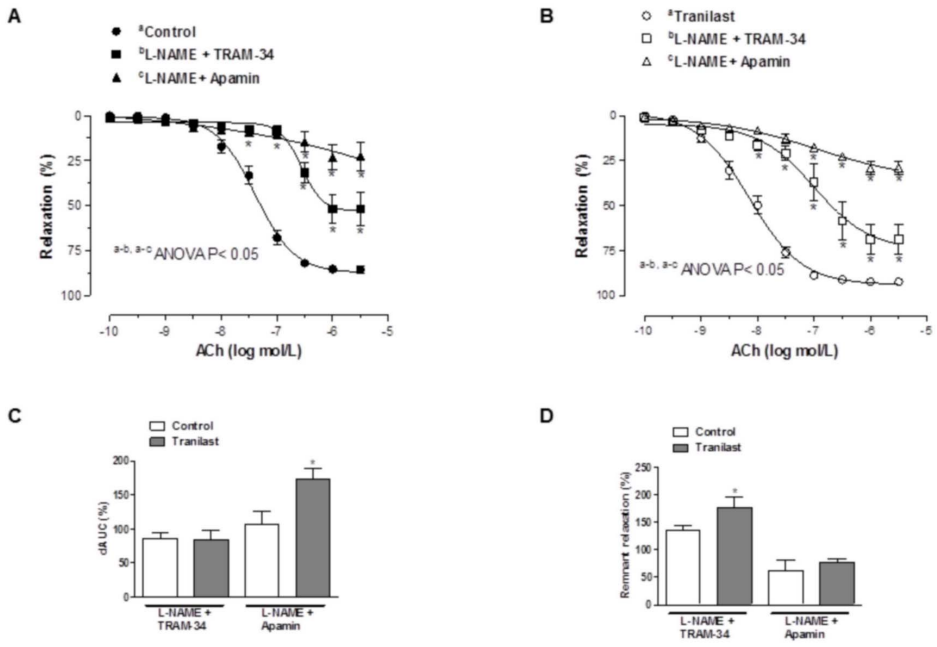

Figure 5. Participation of potassium channels in the vasodilator response to acetylcholine. Effect of preincubation with 100 μmol/L L-NAME plus 1 μM apamin or plus 0.1 μM TRAM-34 on endothelium-dependent relaxation to acetylcholine in noradrenaline-pre-contracted control (A) and tranilast-treated arteries (B). Results are expressed as mean ± SEM. *P<0.05 control vs. tranilast N = 5–7 animals in each group. (C) Differences of area under curve (dAUC) in the absence or presence of 100 μmol/L L-NAME plus 1 μM apamin or plus 0.1 μM TRAM-34. Results are expressed as mean ± SEM. dAUC values are expressed as percentage. *P<0.05 control vs. tranilast. N = 5–7 animals each group. (D) Representation of remnant acetylcholine-induced vasodilation after preincubation with 100 μmol/L L-NAME plus 1 μmol/L apamin or plus 0.1 μM TRAM-34, expressed as mean ± SEM of percentage of AUC. *P<0.05 control vs. tranilast. N = 5–7 animals each group.
doi:10.1371/journal.pone.0100356.g005

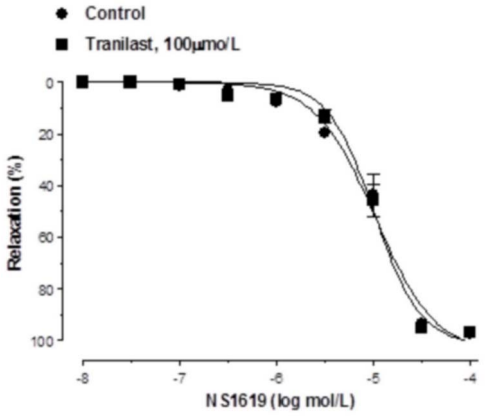

Figure 6. Vasodilator response to K^+-channel openers. Effect of tranilast on the relaxation to the large conductance calcium-activated K^+-channel opener NS1619 in de-endothelized rat mesenteric arteries. Results are expressed as mean ± SEM. N = 5–7 animals in each group.
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rat mesenteric resistance arteries this relaxation is mainly mediated by the release of NO and EDHF [30], but not by COX-derived products [31]. Contradictory effects of tranilast on NO release have been described, since both increases [32], decreases [14,33,34] and no modifications [15] of NO release have been reported in several tissues after tranilast preincubation. Additionally, multiple studies have described an antioxidant effect of tranilast treatment in both *in vivo* and *in vitro* experimental procedures [14,32,33,35–39]. With this in mind, the effects produced by tranilast in ACh-induced vasodilation could be mediated by changes in NO synthesis and/or bioavailability. In order to analyze this possibility, we preincubated control and tranilast-exposed mesenteric resistance segments with the non-specific NOS inhibitor L-NAME. We observed that, after preincubation with this drug, ACh-induced relaxation was decreased to a similar extent in both experimental conditions indicating that NO does not participate in the effect observed after preincubation with tranilast. This was confirmed by the fact that NO release, superoxide anion formation and vasodilator response to NO donor DEA-NO were not modified after preincubation with tranilast, similarly to reported in rat aorta [15]. All these results contrast with our previous results in superior mesenteric artery, where we observed decreases in neuronal NO and superoxide anion releases and an increase in the vasodilator response to DEA-NO after tranilast preincubation [14]. In conclusion, the results obtained in the present study confirm the fact that the increased vasodilator response to ACh produced by tranilast is not due to modifications in the NO pathway.

Hyperpolarizing mechanisms are important regulators of the membrane potential and hence of vessel tone [29], this mechanism being particularly important in small arteries and arterioles. Although controversial, NO has been described to exert a hyperpolarizing role in several vascular beds [40,41]. This hyperpolarization produced by NO can be due to an activation of different potassium channels, including large-conductance calcium dependent potassium channels and voltage-dependent potassium channels [42]. Thus, the effects of tranilast on non-membrane potential-dependent actions of DEA-NO were investigated in arteries preconstricted with a high K^+ solution, thus blocking hyperpolarization by decreasing the plasma membrane potassium gradient [43]. The results showed that, in KCl-precontracted arteries, the vasodilator response induced by DEA-NO was reduced to a similar extent in control and tranilast-incubated mesenteric segments, confirming the hyperpolarizing role of NO in this vascular bed, and also that this effect is not altered by tranilast.

EDHF plays, in addition to NO, an important vasodilator role in resistance vessels. The relaxation induced by EDHF is endothelium-dependent, insensitive to inhibition by a combination of NOS and COX inhibitors, and leads to hyperpolarization of vascular smooth muscle cells [44]. In order to determine whether the increase in ACh-induced vasodilation induced by tranilast is due to an increase in EDHF participation, control and tranilast-incubated mesenteric resistance arteries were precontracted with a high K^+ solution. We observed that, in this experimental condition, vasodilation to ACh was reduced in both control and tranilast-incubated segments, but more markedly in segments exposed to tranilast. Initially, the EDHF-mediated response was attributed to activation of small, intermediate and large conductance calcium-activated K^+-channels, although the participation of the latter has been questioned [45–49]. In presence of a combination of small (SKCa) and intermediate conductance calcium-activated K^+-channel (IKCa) blockers (apamin+TRAM-34, respectively), we also observed a greater inhibition of the ACh-induced vasodilation in tranilast-preincubated segments compared to control segments. However, a differential effect of tranilast on each type of calcium-activated potassium channel must also be considered. The fact that a combined preincubation with L-NAME plus TRAM-34 decreased ACh-induced relaxation

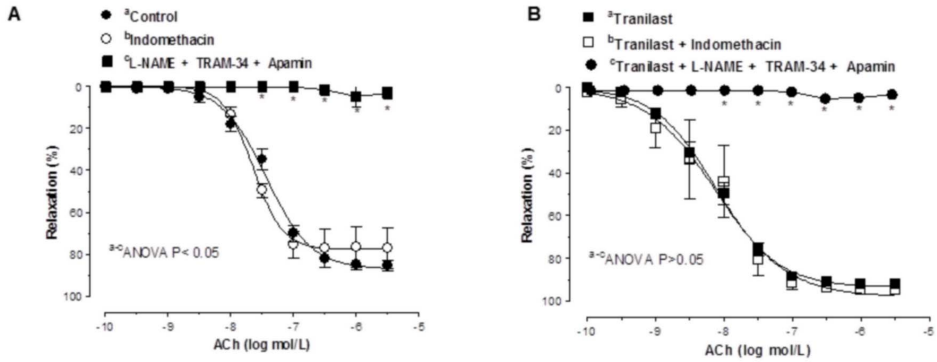

Figure 7. Participation of prostanoids in the vasodilator response to acetylcholine. Effect of preincubation with 10 μM indomethacin or with 100 mmol/L L-NAME plus 1 μM apamin plus 0.1 μM TRAM-34 on the concentration-dependent relaxation to ACh in control (A) and tranilast-treated (B) rat mesenteric resistance arteries. Results are expressed as mean ± S.E.M. *P<0.05 control vs. tranilast. N = 6–7 animals in each group.
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similarly in both experimental conditions, while a combination of L-NAME plus apamin produced a more marked decrease in ACh-induced relaxation in tranilast-incubated segments, suggests a greater participation of SKCa channels through tranilast preincubation. These findings indicate that hyperpolarization produced by EDHF is responsible for the tranilast-mediated effects on the ACh-induced dilation in mesenteric resistance arteries, due to an increased SKCa channel participation after preincubation with tranilast. Since the importance of the hyperpolarizing mechanism in endothelium-dependent relaxations increases as the vessel size decreases [50,51], this result can explain the difference in the effect of tranilast on ACh-induced vasodilation previously observed in superior mesenteric artery and aorta, where the role of EDHF in endothelium-dependent relaxation is essentially absent [14,15].

The greater participation of EDHF in ACh-induced response in tranilast-incubated arteries may be associated to an increase in potassium channel activation by EDHF or to an increase in EDHF generation. The fact that the vasodilation induced by NS1619 (a large conductance calcium-activated K^+-channel opener) was not altered in the presence of tranilast seems to rule out a greater activation of these channels by the tranilast effect. However, we must take into account that these channels are also present in endothelial cells, whose activation alters the release of several vasoactive substances [52–56]. Taken together our results indicate that tranilast increases the vasodilator response to ACh through a mechanism that implicates a greater participation of EDHF. This effect seems to be associated with a greater activation of SKCa channels, without modifying the participation of IKCa channels,

As we have previously reported [31], COX-derived products do not participate in the relaxation induced by ACh in control situations in mesenteric resistance arteries. However, in some pathological situations, such as hyperaldosteronism, we have also described participation by COX-derived products in vascular function, including relaxation to ACh [19,31]. In the present study, the COX inhibitor indomethacin did not affect the relaxation to ACh in the absence or presence of tranilast, confirming the non-participation of COX-derived products in both experimental conditions. The fact that in the presence of L-NAME plus TRAM-34 plus apamin the relaxation to ACh was abolished confirmed this observation, since it demonstrates that the vasodilator response to ACh is only due to NO and EDHF in these experimental conditions.

In summary, tranilast increased the endothelium-dependent relaxation to acetylcholine in rat mesenteric resistance arteries. This effect is independent of the NO and COX pathways but involves EDHF, and is mediated by an increased role of small conductance calcium-activated K^+ channels. Similar alterations in endothelial function in this vascular bed have been associated to altered splanchnic circulation and the development of organ failure [19]. Therefore, these results lead us to consider it important to evaluate the hemodynamic conditions of patients receiving treatment with tranilast.

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Author Contributions

Conceived and designed the experiments: FEX JBR GB. Performed the experiments: FEX JBR ES LC MC. Analyzed the data: FEX JBR ES LC GB. Contributed reagents/materials/analysis tools: FEX JBR GB. Wrote the paper: FEX JBR GB.

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Consideraciones generales

La amplitud de resultados obtenidos en esta Tesis doctoral nos permite realizar una serie de consideraciones generales.

A lo largo de los años 70 diversos estudios histoquímicos y funcionales demostraron la presencia de inervación adrenérgica en arteria mesentérica de rata (Lindquist 1971; Furness y Marshall, 1974). El papel del sistema nervioso simpático en el control de la homeostasis cardiovascular y el control del tono vascular, ha sido desde entonces, ampliamente estudiado, así como su implicación en el desarrollo de enfermedades cardiovasculares (Seravalle y cols., 2014). El neurotransmisor principal de la inervación simpática es el vasoconstrictor NA. A partir de mediados de los años setenta diversos datos indicaron la existencia en las terminaciones nerviosas adrenérgicas de los cotransmisores ATP con un efecto vasoconstrictor y NPY con una función moduladora (Burnstock G 1987, 1995)

La presencia de la denominada inervación no adrenérgica no colinérgica (NANC) en diferentes lechos vasculares de distintas especies fue descrita en los años 70 y 80 y se postularon múltiples sustancias como mediadores de este tipo de inervación (Toda 2003). En este sentido en 1990 surgió la hipótesis de que el NO actuaba como neurotransmisor vasodilatador en arteria cerebral de perro (Toda 1975). Aunque durante años se cuestionó la función de la inervación nitrérgica en distinto lechos vasculares incluyendo el mesentérico (Toda 2003; Amerini y cols., 1993), resultados previos de nuestro grupo y los obtenidos en esta tesis doctoral demuestran la presencia de inervación nitrergica funcional en las distintas cepas de rata analizadas, ya que en presencia de L-NAME la respuesta vasoconstrictora inducida por EE se incrementa entre un 25-30%. La discrepancia en los resultados referidos por los distintos investigadores puede deberse a diferencias experimentales. Mientras que en nuestro caso la EE se aplica sobre la arteria con un tono similar al fisiológico y en ausencia de tratamiento farmacológico, estos autores utilizan bloqueantes ganglionares y la EE se aplica en las arterias precontraídas con una concentración elevada de agonistas α-adrenérgicos. De acuerdo con los resultados obtenidos en la arteria peneana, esta metodología condicionaría la función de la inervación nitrérgica, disminuyendo la liberación de NO, ya que se ha descrito que su liberación neuronal se

inhibe presinápticamente a través de receptores α2-adrenérgicos (Simonsen y cols.,. 1997) Nuestra observación de que la participación del NO neuronal disminuya a medida que incrementamos la frecuencia de EE, en la cual se libera una cantidad de NA dependiente de frecuencia, refuerza esta hipótesis. Recientemente, los mismos autores que negaron la existencia de inervación nitrérgica funcional en esta arteria, han reconocido su presencia y participación en el desarrollo de hipertensión espontánea e hipertensión portal (Toda 2014).

Mediante la perfusión del árbol mesentérico, diversos autores han puesto de manifiesto la existencia de inervación sensitiva, cuyo neurotransmisor principal es el péptido vasodilatador CGRP (Kawasaki y cols., 1988; 1991; Ralevic y Burnstock, 1996; Li y Duckles, 1992), y su implicación en diversas situaciones fisiopatológicas como la edad, gestación e hipertensión. Estos resultados han sido obtenidos mediante la aplicación de EE en esta arteria precontraída, produciendo la liberación de CGRP y consecuente vasodilatación. Nuestros resultados, obtenidos aplicando la EE sin tono previo, confirman que en arteria mesentérica superior se libera CGRP (Artículo 3), aunque no observamos vasodilatación. La escasa relajación inducida por CGRP aplicado de forma exógena nos indica que esta arteria tiene inervación sensitiva con el CGRP como neurotransmisor, y que la cantidad que se libera en condiciones control es insuficiente para producir una vasodilatación valorable. Sin embargo, hemos observado su liberación y consecuente efecto vasodilatador en distintas situaciones patológicas (Blanco-Rivero y cols., 2011; del Campo y cols., 2004 y Artículo 3). Estos resultados están de acuerdo con las conclusiones de una exhaustiva revisión en la que se indica que a pesar de existir una amplia distribución de este tipo de inervación en los distintos vasos sanguíneos, su participación sólo se pondría de manifiesto en eventos cardiovasculares adversos como un mecanismo compensador o protector (Russell y cols., 2014).

Mecanismos implicados en las modificaciones de la función perivascular en las distintas situaciones analizadas

La tabla 1 muestra un resumen de los mecanismos implicados en la modificación de la función de la inervación perivascular mesentérica.

Tabla 1: Resumen de los mecanismos implicados en las alteraciones de función perivascular mesentérica en las distintas situaciones analizadas. Los símbolos ↑, ↓ e = representan incremento, disminución y no modificación, respectivamente. El símbolo — presente en la columna de la inervación sensitiva representa que ha sido analizado y se ha observado que no participa en la respuesta. La ausencia de análisis se representa con espacios en blanco.

			Resp. Induc. por EE	Simpática			Nitrérgica (NO)				Sensitiva (CGRP)		Resp. a ACh
				NA	ATP	Resp.	Liber.	Resp.	Expresión (nNOS)	Activación (P-nNOS)	Liber.	Resp.	
Embarazo			↓	↓		↓	↑	=	=	↑	—	—	=
Lactancia			↑	=	↑	=	↓	=	↓	↓	—	—	=
Células Cebadas	LPS	2h	↑	=		↑	↓	=	=	↓			
		5h	=	=		=	↓	↑	=	↓			
	KETOTIFEN		↑	=		=	↓	↑	=	↓			=
	TRANILAST		↓	=		↓	↓	↑	=	↓			=
HP	STHP		↓	↓		=	↑	=	=	↑	↑	↓	
	LTHP		=	=		=	=	=			—	=	
Ejercicio Físico Moderado	SHR		↓	↓		↑	↑	↑	↑		=	=	
	HFD		↓	↓	↑	=	↑	=	↑	↑	—	—	↑
Programación Fetal			↑	↑	↑	=	↑	=	=	↑	—	—	↓

Respuesta vasoconstrictora inducida por EE (Resp. Induc. por EE), Respuesta (Resp.), Liberación (Liber.), Hipertensión portal (HP), A corto plazo (STHP), A largo plazo (LTHP), Ratas espontáneamente hipertensas (SHR), Ratas alimentadas con dieta rica en grasa (HFD)

La respuesta vasoconstrictora inducida por EE es el resultado integrado de los distintos tipos de inervación. Aunque la respuesta neta se incremente o disminuya, los mecanismos que participan en estas modificaciones dependen de la situación analizada.

Influencia del endotelio

El endotelio modula la respuesta a diferentes sustancias vasomotoras (Vanhoutte, Houston, 1985 Ralevic 2002) y su eliminación produce un incremento en las respuestas a diferentes agentes vasoconstrictores, incluida la NA (Moncada y cols., 1991; Dora y cols., 2000). Nuestros resultados indican que en los modelos experimentales en los en los que la respuesta vasodilatadora a ACh no se modifica, como durante la gestación (Artículo 1), lactancia (Artículo 2) y efecto de fármacos estabilizadores de células cebadas (Artículo 8), el incremento en la respuesta vasoconstrictora inducida por EE en ausencia de endotelio, lógicamente es similar a sus respectivos controles experimentales. Sin embargo, es llamativo que en animales alimentados con una dieta rica en grasa (Artículo 6) y en descendientes adultos de madres diabéticas (Artículo 4) en los que hemos observado disfunción endotelial, se incrementó la respuesta contráctil inducida por EE en la misma medida que en sus respectivos controles.

Esta carencia de efecto sugiere que la disminución en la respuesta vasodilatadora a ACh observada no es suficiente para inducir cambios en la respuesta de origen neuronal, ya que existe una diferencia cuantitativamente significativa entre la disfunción endotelial y la ausencia total de endotelio.

Interacciones entre los distintos tipos de inervación

Se ha descrito una interacción entre los distintos tipos de inervación en diferentes lechos vasculares, incluido en mesentérico (Kawasaki y cols., 1990a; Kawasaki y cols., 1990b; Hatanaka y cols., 2006; Kawasaki y cols., 2011).

La mayor parte de los resultados publicados indican que el NO inhibe la liberación de NA (Kawasaki y cols., 2011; Macarthur y cols., 2011), aunque también se ha descrito que el NO de origen neuronal la aumenta (Wang y Golledge, 2013). Establecer inequívocamente la existencia o no de interacciones entre la inervación simpática y nitrérgica es compleja ya que su función está regulada por neurotransmisores moduladores (Macarthur y cols., 2011) cuya modificación no podemos descartar.

Nuestros resultados, resumidos en la tabla 2, muestran un incremento en la liberación de NA simultáneamente a una disminución en la liberación de NO neuronal en ratas alimentadas con una dieta rica en grasa (Artículo 6) así como una disminución en la liberación de NA simultáneamente a un incremento en la liberación de NO en hipertensión portal (Artículo 3) y durante la gestación (Artículo 1). Estos resultados parecen confirmar una interacción entre ambos tipos de inervación en este lecho vascular.

Tabla 2: Resumen de las modificaciones en la liberación de NA y NO en arteria mesentérica superior en diferentes situaciones fisiopatológicas.

	Liberación de NA	**Liberación de NO**
Embarazo	↓	↑
Hipertensión Portal	↓	↑
Dieta rica en grasa	↑	↓
Lactancia	=	↓
LPS	=	↓
Ketotifen/Tranilast	=	↓
SHR	↑	↑
Descendencia Diabetes	↑	↑

Sin embargo durante la lactancia (Artículo 2) y el tratamiento con LPS (Artículo 7), ketotifen o tranilast (Artículo 8) observamos una disminución en la función nitrérgica mientras que la liberación de NA permanece inalterada. En el modelo de hipertensión en descendencia de madres diabéticas (Artículo 4) y SHR (Marín y cols., 2000) se observa un incremento simultáneo en la función de la inervación adrenérgica y nitrérgica. Estos resultados nos llevan a sugerir que las alteraciones observadas en las distintas situaciones fisiopatológicas en la función de ambas inervaciones no son fruto de una interacción entre las mismas.

Conclusiones

Los resultados obtenidos en esta tesis doctoral demuestran que:

1. Las modificaciones en la función perivascular participan en la adaptación del organismo durante la gestación y la lactancia:

- En la gestación disminuye la respuesta contráctil inducida por EE. Este efecto está mediado por una disminución tanto en la liberación de NA como en la respuesta vasoconstrictora a este neurotransmisor, junto con incremento en la liberación de NO neuronal mediado por una mayor activación de nNOS, mientras que la respuesta vasodilatadora al DEA-NO no se modifica La inervación sensitiva no presenta un papel funcional en este modelo.
- Durante la lactancia se produce un incremento en la respuesta vasoconstrictora inducida por EE. Este incremento está mediado por un incremento en la liberación de ATP, mientras que no se modifica el componente adrenérgico; junto con una diminución tanto en la expresión como en la actividad de nNOS y consecuente liberación de NO, mientras que la respuesta a DEA-NO es similar. No se observa participación ni modificación en la inervación sensitiva.

2. En la vasodilatación esplácnica asociada a la hipertensión portal a corto plazo participa la inervación perivascular disminuyendo la respuesta contráctil inducida por EE. Este efecto se debe a una disminución en la liberación de NA y al incremento tanto en la liberación de NO neuronal mediado por un aumento en la actividad de nNOS, como en la liberación de CGRP. Las respuestas vasomotoras a los distintos neurotransmisores no se modifican. Sin embargo, a largo plazo hay una adaptación en la función neuronal.

3. En la hipertensión desarrollada por la descendencia adulta de madres diabéticas participa la inervación perivascular. En estos animales se incrementa la respuesta vasoconstrictora inducida por EE, este efecto es el resultado neto del incremento en la liberación de los vasoconstrictores simpáticos NA y ATP y de un aumento en la liberación de NO neuronal mediado por un aumento en la activación de nNOS, mientras que las respuestas vasomotoras a NA y DEA-NO no se modifican. La inervación sensitiva no presenta un papel funcional en este modelo experimental.

4. El ejercicio físico moderado es capaz de revertir las alteraciones en la inervación perivascular en hipertensión y prevenirlas en un modelo de obesidad inducido con dieta

grasa, por lo que en los efectos beneficiosos del ejercicio físico también está implicada la regulación neuronal del tono vascular:

- En SHR disminuye la liberación de NA e incrementa su respuesta contráctil, y se incrementa tanto la liberación de NO neuronal, mediado por una mayor expresión de nNOS, como su biodisponibilidad. La inervación sensitiva participa en la respuesta contráctil aunque no se modifica con el ejercicio.
- En ratas sometidas a una dieta rica en grasa disminuye la liberación de NA y aumenta la de ATP; y se incrementa la liberación de NO debida a un aumento tanto en la expresión como en la actividad de nNOS. Las respuestas vasomotoras a NA y DEA-NO no se modifican. La inervación sensitiva no presenta un papel funcional en este modelo experimental

5. El LPS parece desencadenar en una fase temprana un efecto compensador al estado de vasodilatación característico de la sepsis, la cual no se mantiene a largo plazo. A corto plazo, 2 horas, se incrementa la respuesta vasoconstrictora inducida por EE mediada por una menor liberación de NO neuronal mediada por una disminución en la actividad de nNOS, y una mayor respuesta vasoconstrictora a NA. A largo plazo, 5 horas, se mantiene la disminución en la liberación de NO y se restaura la función adrenérgica.

6. El ketotifen y tranilast, fármacos que estabilizan la membrana de las células cebadas, alteran la respuesta contráctil inducida por EE, de forma opuesta. El ketotifen la incrementa a través de la disminución en la actividad de nNOS y por tanto en la liberación de NO neuronal, mientas que no modifica la función adrenérgica. El tranilast disminuye la respuesta vasoconstrictora disminuyendo tanto la liberación de NO como la respuesta vasoconstrictora a NA.

7. En vasos de resistencia el tranilast también contribuye a facilitar un estado de vasodilatación, ya que incrementa la relajación dependiente de endotelio por una mayor mayor participación de EDHF cuyo efecto está mediado a través de canales de potasio dependientes de calcio de baja conductancia, descartándose la participación del NO y prostanoides derivados de COX. Por lo que, la elección de uno u otro fármaco estabilizador puede tener efectos hemodinámicos.

Estos datos tomados en su conjunto nos permiten realizar diversas consideraciones generales:

- La existencia de inervación nitrérgica funcional en el control del tono vascular en la arteria mesentérica superior de todas las cepas analizadas.
- La inervación sensitiva no participa en la respuesta contráctil inducida por EE en situaciones fisiológicas, mientras que en las distintas situaciones patológicas analizadas sólo contribuye a la vasodilatación esplácnica asociada a la hipertensión portal.
- Los mecanismos por los cuales se incrementa o disminuye la respuesta vasoconstrictora inducida por EE no son homogéneos y difieren en función de la situación experimental analizada.
- El incremento en la respuestas vasoconstrictora inducida por la liberación de los distintos neurotransmisores en arteria mesentérica superior no se ve afectada por la disfunción endotelial..
- No parece existir una interacción entre la inervación nitrérgica y adrenérgica.

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